COMPARISON OF IRON OXIDATION BY ACIDOPHILIC BACTERIA

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ABSTRACT

A range of obligate acidophilic iron oxidising bacteria were compared physiologically, kinetically and biochemically. The organisms were mesophiles, Thiobacillus ferrooxidans and Leptospirillum ferrooxidans, moderate thermophiles designated strains ALV, BC1, LM2, TH1 and TH3 and thermophile Sulfolobus BC65. Each organism retained iron oxidising activity in non-growing cell suspensions. Measurement was made of apparent K_m and K_i for ferrous iron oxidation and it's inhibition by ferric iron in these suspensions. Values were derived from three graphical representations of the data. Values differed where identical strains had been previously reported. Evidence of a fixed relationship between K_m and K_i suggested that these differences were derived from experimental variation. Ferric iron was a competitive inhibitor of iron oxidation but copper was an uncompetitive inhibitor of L.ferrooxidans. Responses of cells to ferric iron during growth indicated the predictive power of suspension kinetics, extremes in batch culture coinciding with the lowest and highest measured values of K_i. Continuous culture provided evidence of the relevance of this data to growth and explained relative cell numbers during competition in mixed mesophilic culture. These could also explain previously reported observations in mineral cultures.

The production of ferric iron was controlled both by pH and process design with T.ferrooxidans. Comparable production was provided with L.ferrooxidans, utilizing it's ability to form macroscopic cell aggregates in sub-optimal conditions. Optimum growth conditions varied nutritionally with each strain and with particle size, ore mineralogy and carbon dioxide concentration during mineral dissolution by Sulfolobus BC65. The sulphur requirement for growth was strain dependent, quantitatively and qualitatively. Strain ALV indicated that reduced sulphur was not an obligate requirement for thermophilic iron oxidation. Iron oxidation appeared to be the controlling factor in mineral dissolution at 68°C. Iron oxidation was limited prior to maximum target metal release.

Based on optical spectra each organism contained a range of (different) respiratory chain components. Both L.ferrooxidans and Sulfolobus BC65 had absorbance maxima not attributable to known cytochrome species. The peak at 578 nm for L.ferrooxidans was due to a red-pigmented, acid stable soluble protein which was reduced by ferrous iron.

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1 BACTERIAL MINERAL AND IRON OXIDATION

Acid water in runoff from mining operations was associated with soluble sulphate from the oxidation of mineral sulphides. Investigation of the pollution aspects of runoff from coal mining identified bacteria as causative agents of sulphide oxidation (Leathen, Braley and McIntyre, 1953; Temple and Delchamps, 1953). The first strains of such bacteria isolated were described as Thiobacillus ferrooxidans. Being aerobic, mesophilic rods these were capable of using inorganic metal transformations as sources of energy (Colmer and Hinkle, 1947). Physiologically, Thiobacillus ferrooxidans was shown capable of ferrous iron oxidation under autotrophic conditions (Temple and Colmer, 1951). Obligately aerobic, the cell carbon was derived from atmospheric carbon dioxide, fixation often controlled by the growth conditions.

The basic reaction of iron oxidation was shown to be pH dependant. In an aqueous sulphate medium at low pH ferrous iron oxidized, slowly. At the same pH, the rate of this reaction was increased by up to six orders of magnitude in the presence of T.ferrooxidans (Lacey and Lawson, 1970). The stoichiometry of the reaction is shown in equation 1.1 (Silverman and Lundgren, 1959). At pH 4 auto-oxidation of the ferrous iron occurred. This rapid loss of iron as a ferric hydroxide prevented further growth (Lundgren, Vestal and Tabita, 1972).

$$4\ FeSO_4 + O_2 + 2H_2SO_4 \longrightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2O \qquad 1.1$$

1.1 CHEMISTRY OF IRON OXIDATION AND IT'S ROLE IN MINERAL DEGRADATION

Investigation provided the evidence of a direct role for

T.ferrooxidans in mineral solubilization. This, for pyrite was an acidogenic reaction. Associated with the solubilization, iron was released in the ferrous form (equation 1.2) due to cell interaction with the mineral surface (Bryner and Jameson, 1958; Silverman, 1967). The released ferrous iron was then oxidized according to equation 1.1

$$2\ FeS_2 + 7O_2 + 2H_2O \longrightarrow 2\ FeSO_4 + 2\ H_2SO_4 \qquad 1.2$$

In both reactions, the final product was ferric sulphate. Ferric iron as an oxidant, was capable of the chemical, inorganic oxidation of pyrite, at pH 2. The reaction medium had always to be strongly acidic to maintain the (ferric) iron in solution, and hence active (Woodcock, 1961). Elucidation of the chemistry of mineral dissolution was developed into a theory of a dual mechanism for bacterial mineral dissolution (Silverman, 1967; Smith and Shumate, 1970). Operating simultaneously, the dual mechanism under acidic conditions was composed of a direct cell-mineral lattice interaction, and the chemical ferric iron attack. The ferric iron attack was indirectly of biological origin through the bacterial re-oxidation of ferrous iron produced (equation 1.3). Indirect attack was not exclusive to iron sulphides, following the reaction generalized in eq 1.3, for metal sulphides(MeS),(Bryner, Beck, Davis and Wilson, 1954; Ivanov, Nagirnyak and Stepanov, 1961). Energy derived from bacterial sulfide oxidation was not exclusive to the sulfide moiety and could be

$$MeS + Fe_2(SO_4)_3 \longrightarrow MeSO_4 + 2\ FeSO_4 + S^o \qquad 1.3$$

derived from oxidation of the metal moiety, including reduced forms of non-iron species such as uranium and copper (1.3.1). Combined, equations 1.1 and 1.2 illustrated that the oxidative products of the overall reaction included net production of sulphuric acid

(equation 1.4). This both kept the reactive

$$4\ FeS_2 + 15O_2 + 2H_2O \longrightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4 \quad 1.4$$

iron species in solution and pinpointed the source of acidity in acid mine drainage. Elemental sulphur formed (eq 1.3) was also an oxidizable energy source, forming additional sulphuric acid (equation 1.5). Bacterial mineral dissolution hence caused increasing acidity with progression of the reaction in the environment.

$$2S^\circ + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4 \quad 1.5$$

1.2 BACTERIAL ATTACHMENT AND DIRECT LEACHING

Indirect oxidation could be shown given pyrite oxidation by ferric iron but evidence of a direct attack had to involve a different mechanism of breakdown, beyond the observations of microbial attachment to mineral surfaces (Gormley and Duncan, 1974). The corrosion of mineral surfaces associated with bacterial leaching was dependent on the crystallographic orientation of the mineral. The actual positioning of the cells on the mineral surface was also related to specific orientations in the mineral matrix. Concomitant to this, bacterial attachment was selective to the sulphide phases of an ore, but was not ubiquitously present at all points of attack (Barry and Murr, 1978). With pure mineral crystals no selective (bacterial) attachment was observed, yet the etching patterns for oxidative corrosion differed in the presence of bacteria (Keller and Murr, 1982). These etching patterns however did not correspond to patterns of bacterial attachment only to bacterial presence (Keller and Murr, 1982). Predictive growth models of T.ferrooxidans on mineral sulphide assumed that growth required attachment. Adapted growth models predicted a role for both attached and unattached bacteria, with quantified estimations of the size of colonised areas that

agreed with the concept of selective attachment sites in minerals (Chang and Myerson, 1982). Absence of attached bacteria did appear to reduce sulphur oxidation associated with pyrite dissolution (Arkestyn, 1979). This related to elemental sulphur oxidation, also released as a product of ferric iron leaching (eq 1.3). Therefore the evidence for direct sulphur removal from the mineral exclusively by bacteria was not shown.

1.3 NUTRITIONAL REQUIREMENTS OF T.ferrooxidans

The physiology and biochemistry of T.ferrooxidans were studied to aid elucidation of the process of mineral dissolution. How did the parameters and mechanisms controlling both iron and sulphur oxidation affect subsequent leaching processes and vice versa?

1.3.1 Energy Source

T.ferrooxidans was capable of chemolithotrophic growth, solely based on the sulphur oxidation of eq 1.3 (McGoran, Duncan and Walden, 1959). Additionally sufficient energy could be provided by the oxidation of partially reduced sulphur compounds such as thiosulphate and tetrathionate (Colmer, 1962; Hutchinson, Landesman, Duncan and Walden, 1966) and trithionate (Tuovinen and Kelly, 1974). Based on the physiological reaction to such inorganic substrates the mesophilic strains of acidophilic iron and sulphur oxidising bacteria were separated into three species, T.ferrooxidans , Ferrobacillus ferrooxidans and Ferrobacillus sulfooxidans. This was supported by kinetic studies (Bounds and Colmer, 1972). Each individual species was still identified with the original problem of acid mine drainage (Dugan and Lundgren, 1964). The genus Ferrobacillus was disputed and the recommendation, based on further physiological comparison that all three bacterial strains be known under the binomial of T.ferrooxidans (Kelly and Tuovinen, 1972) was accepted. The physiology represented by T.ferrooxidans was subsequently identified worldwide,in association with

exposed metal deposits (Gentina and Acevedo, 1985).

The range of sulphides oxidizable by the combined T.ferrooxidans strains was identified as being both diverse and unlimited to iron containing minerals. These ores included sources of copper, nickel, arsenic, lead and uranium (Brierley,C.L, 1978; Lundgren and Silver, 1980). Although the sulphide moiety was associated with being the energy source for the organism, T.ferrooxidans was implicated in direct metal oxidations, of ionic species other than ferrous iron. These included reduced copper and tin species (Lewis and Miller, 1977) and reduced forms of uranium (DiSpirito and Tuovinen, 1982).

1.3.2 Carbon sources

Energy derived from the oxidation of inorganic species was used in the fixation of atmospheric carbon dioxide (CO_2). The CO_2 supplied the complete carbon requirement for T.ferrooxidans cells. Assimilation, driven by ferrous iron oxidation was via the Calvin-Benson cycle (Gale and Beck, 1967). The same CO_2 fixation was driven by the oxidation of sulphur (reduced and elemental forms) and sulphide minerals. Nearly 80% of the energy from ferrous iron oxidation was used to drive the Calvin cycle (Kelly, 1978). This energy intensive metabolism resulted in low cell yields compared to the amount of energy source oxidised, relative to heterotrophic metabolism. The energy consumed was directly related to production of reduced pyridine nucleotides. As with CO_2 assimilation the energy required for the necessary reversal of the electron transport chain was available from both sulphur compounds and ferrous iron (Aleem, Lees and Nicholas, 1963).

Glucose, present at 10% w/v reduced the degree of oxidation by T.ferrooxidans by 5 to 15% for sulphur and 40 to 50% for ferrous iron (Silver, Margalith and Lundgren, 1967). The use of glucose as a carbon and energy source was though observed for strains of T.ferrooxidans. This transition to heterotrophic metabolism reduced the capability for iron oxidation, which was completely lost after long periods of heterotrophic adaptation (Tabita and

Lundgren, 1971; Shafia and Wilkinson, 1969). This change in nutrition was matched by changes in the method of carbon metabolism (Kelly, 1978). An heterotrophically adapted strain, incapable of iron oxidation was renamed Thiobacillus acidophilus. This strain differed from the original culture in the G+C% ratio of it's DNA (Guay and Silver, 1975). Investigation of the DNA in these changing cultures led to the conclusion that some of the reports of heterotrophy were due to contaminant organisms closely associated with the "host" T.ferrooxidans culture (Tuovinen,Kelly, Dow and Eccleston, 1978).

Thiobacillus acidophilus was facultatively heterotrophic obtaining energy from either sulphur or glucose but not ferrous iron. Subsequently, heterotrophs were isolated from several culture collection strains of T.ferrooxidans (Arkesteyn and De Bont, 1980; Johnson and Kelso, 1983). Though some were classified as T.acidophilus these strains were all obligate heterotrophs, incapable of sulphur oxidation and were subsequently classified as strains of Acidophilium (Harrison, 1981; Harrison, 1984). Heterotrophic contaminants could be removed from T.ferrooxidans by plate culture, via serial dilution (Harrison, 1984) or by incorporation of copper into the growth medium (Johnson and Kelso, 1983). Copper, at 1g to 5g per litre was toxic to the Acidophilium strains, but the T.ferrooxidans strains displayed inherent resistance. Removal of heterotrophs removed the ability of reputedly pure T.ferrooxidans cultures to utilise glucose as a sole carbon source. One strain, checked for heterotrophs, unable to use glucose as a sole energy source, could utilise glucose in the presence of ferrous iron (Barros, Rawlings and Woods, 1984). The rate of iron oxidation was reduced during this mixotrophy compared to oxidation in the absence of glucose. This was the only true mixotrophic strain reported.

1.3.3 Inorganic nutrients

Utilised as an energy source in it's reduced forms, sulphur was incorporated by T.ferrooxidans cells in the fully oxidised form of sulphate. Sulphate was therefore

introduced into the composition of experimental media as the most efficacious acidifying agent, in the form of sulphuric acid. This avoided inhibition by other anionic species (1.4.1). Assimilated during ferrous iron supported growth, sulphate was not assimilated for cellular biosynthesis during thiosulphate supported growth although the enzymes for sulphur reduction were still present (Tuovinen, Kelley and Nicholas, 1976). The sulphur requirement was met by the thiosulphate. Continued growth on reduced sulphur compounds required that T.ferrooxidans be supplemented with trace amounts of iron (Eccleston and Kelly, 1978).

Nitrogen was assimilated from ammonium, though the cell did contain the enzymes for direct fixation of dinitrogen (Mackintosh, 1978) and could incorporate this into cell material. Ammonium was the preferred nitrogen source, as measured by iron oxidation. One strain was capable of nitrate assimilation (Tuovinen, Panda and Tsuchiya, 1979). In the absence of this added nitrogen, oxidation of iron could still proceed without associated growth. Replacement of ammonium salts was successfully achieved with urea during growth on chalcopyrite. The presence of urease was detected in the cell but not excreted (Lapido, Toro, Paponetti and Cesare, 1988).

Phosphate and trace metals were also essential nutrients for T.ferrooxidans (Tuovinen, Niemela and Gyllenberg, 1971). These inorganic nutrients had to be provided in culture media but were largely present in natural environments in association with ore bodies. More concentrated nutrients, as inorganic salts were required for growth on mineral sulphide than on ferrous iron. This reflected the greater potential energy supply locked in the mineral.

1.4 CULTURAL INFLUENCES ON GROWTH. IONS IN SOLUTION

The external environment had to be strongly acidic to maintain iron species in solution (1.1). This contrasted with the cytoplasmic biochemistry of T.ferrooxidans where

the internal pH was at pH 6.5. (The cell maintained this pH gradient across the cytoplasmic membrane as a component of the proton-electrochemical potential of the cell ,Ingledew, 1982). The acidity also influenced solubility of other heavy metal cations present in the localised environment. The relationship of total solubilised ionic species to the activity of acidophilic bacteria was studied. Effects on both growth and iron oxidation were attributable to the influence of anions as well as cations. With batch culture systems and washed cell suspensions T.ferrooxidans could oxidise iron in the absence of cell growth (Kelly and Jones, 1978), but ionic influences still operated in such growth uncoupled systems.

1.4.1 Anions/ organic acids

During growth on mineral sulphides T.ferrooxidans was most exposed (anionically) to heavy concentrations of sulphate, as the fully oxidised form of the sulphide moiety. Under conditions of sulphate dominance, iron oxidation was more advanced than in environments saturated with either chloride or nitrate anions (Alexander, Leach and Ingledew, 1987). Chloride and nitrate were active inhibitors of iron oxidation. Such inhibition was a general feature of anions, other halides such as iodide and bromide also reducing extent and rate of iron oxidation.

The transfer of such anions across the cytoplasmic membrane resulted in internal acidification of the cytoplasm. Alteration of the pH gradient disrupted the process of electron transfer and therefore of energy generation within the cell. This mechanism for reducing oxidative capacity was proposed as the means by which organic acids, which accumulated in the cytoplasm, reduced iron oxidation (Alexander, et al, 1987). Salt inhibition particularly with sodium chloride, therefore, reduced the observed environmental range of iron oxidising organisms, particularly with reference to marine environments (Cameron, Jones and Edwards, 1984). The presence of organic solvents also reduced the oxidative ability of T.ferrooxidans (Torma and Itzkovitch, 1977). When growing

on pyrite this required the removal of such materials (often present as a by-product of mineral processing) before the re-cycling of solvent-stripped leach liquors to actively oxidizing cultures.

Inhibition of iron oxidation by organic compounds was studied as preventative and remedial operations in the control of acid mine drainage.

Using a series of organic acids the inhibition of both ferrous iron and elemental sulphur oxidation increased with the increasing electronegativity of the organics. Both cell growth and growth uncoupled oxidation of iron and sulphur were inhibited by organic materials (Tuttle and Dugan, 1976). Direct inhibition was observed by the addition to the acidogenic environment of anionic detergents such as sodium lauryl sulphate (Dugan and Apel, 1983) and linear alkylbenzene sulfonate (Dugan, 1975). The presence of low molecular weight organic acids caused cell leakage but precipitates of sodium benzoate and potassium sorbate had no direct action against iron oxidizing bacteria until acidogenic conditions prevailed. This resulted in the formation of sorbic and benzoic acids, reducing further oxidation (Onyska, Klainmann and Erickson, 1984). The efficacy of such compounds was related to their specific action against acidogenic bacteria, without wide spectrum toxicity in the environment and with required inhibition at low concentrations (Dugan and Apel, 1983).

Values of pH also affected the degree of inhibition by oxyanions of sulphur. Growth, inhibited by thiosulphate and tetrathionate, was associated with pH values below the optimum for growth on these compounds (Eccleston and Kelly, 1978). Batch cultures with 120 mM ferrous iron were completely inhibited by 10 mM thiosulphate, sulfite, metabisulfite and tetrathionate. Toxicity, greatest with thiosulfate and metabisulfite (both completely inhibitory at 1 mM concentration) was greater at pH 1.5 than pH 2.5. Unstable compounds in acid, their toxicity was due to degradation products (Hurtado, Tsai and Tuovinen, 1987). They reduced cytochrome species, suggesting that

inhibition was related in some way to the respiratory chain.

1.4.2 Metal toxicity

The major cationic species present in mineral leaching environments were those formed on the solubilization of heavy metals. Of the base metals, T.ferrooxidans was tolerant to concentrations greater than 10g per litre. Bacterial activity was noted in adapted strains at copper concentrations of 55g per litre, nickel concentrations of 50g per litre and zinc concentrations of 112g per litre(Sakaguchi, Silver and Torma, 1976; Bosecker, 1977; Torma, Walden, Duncan and Branion, 1972). The greater toxicity of copper than zinc was confirmed when bacterial activity, almost negligible with 80g per litre copper sulphate was little affected in the presence of 80g per litre zinc sulphate. The concentration of zinc required to kill the cells was 160g per litre. Adaptation raised the upper limits of ferrous iron oxidation. The co-presence of 40g per litre zinc halted the lag phase induced by 40g per litre copper in unattenuated strains. In contrast, low concentrations of soluble silver were toxic to T.ferrooxidans (Hoffman and Hendrix, 1976). Inhibition occurred at 50mg per litre (50 ppm) and prevented growth in ferrous iron medium with some strains at below 0.1 ppm. This severe inhibition progressed from increases in the length of the lag phase during iron oxidation with increasing amounts of silver nitrate added (Norris and Kelly, 1978). The length of lag phase due to inhibition was pH dependent, being greater with increased acidity. Silver uptake by cells was also proportional to it's concentration. This was associated with the loss of magnesium and potassium from the cells. The addition of 50 mM potassium slightly reduced the lengths of lag phase during inhibition (Norris and Kelly 1978). Adaptation to resist toxic concentrations resulted in a strain able to oxidize ferrous iron and grow with 5×10^{-4} M silver present (Sugio, Tano and Imai, 1981). Toxicity to the cell was though dependent on the solubility of silver, capable of complexation and co-precipitation in culture media (Dutrizac and Kaiman, 1976). This reduced it's

availability to cause inhibition.

Silver could be directly added as a catalyst in microbial chalcopyrite leaching (Lawrence, Vizsolyi and Vos, 1985). During stirred tank leaching the inocula were used successfully pre-incubated in the presence of 1mM silver (Ehrlich, 1988). This was investigated given that the sulphide moiety reported in suspension as elemental sulphur, not oxidized sulphate. Thiosulphate complexed the silver reducing the concentration remaining in solution below that expected to cause inhibition. Yeast-extract, at 0.02% w/v reduced the lag phase in cultures inhibited by silver. The use of silver as a catalyst could be balanced against it's known toxicity by composition of the media thereby removing it from solution via complexation with certain media constituents (Tuovinen, Puhakka, Hiltunen and Dolan, 1985).

Other metals displayed inhibitory effects. With cobalt sulfide as substrate, all the tested strains grew with 0.1 M cobalt but 0.2 M cobalt lengthened the lag phase by several days, dependent on strain (Imai and Sugio, 1983). In inhibition studies, the toxicity was always strain specific quantitatively, at the lower concentrations. Uranium toxicity which could be as low as 1 mM was decreased by selective subculturing with uranyl ions (Tuovinen and Kelly, 1974a), thereby increasing resistance. One isolate taken from a uranium mine site, but without artificial adaptation grew without inhibition in the presence of up to 4 mM UO_2^{2+}. Resistance appeared to be associated with the occurrence of plasmids in these strains (Martin, Dugan and Tuovinen, 1983).

Mercury was found to be highly toxic, although volatilization could occur (Olson, Porter, Rubinstein and Silver, 1982). Sensitive strains oxidized iron in the presence of 0.1 ppm Hg but only resistant strains were able to grow with 0.5 ppm (Baldi and Olson, 1987). Resistance was supplied by a mercuric reductase enzyme. In contrast, arsenic could be tolerated at up to 17g per litre (Kandemir, 1984).

1.5 PHYSICO-CHEMICAL PARAMETERS INFLUENCING GROWTH AND MINERAL OXIDATION BY T.ferrooxidans

1.5.1 Pulp density and gas transfer

With the nutritional requirements of T.ferrooxidans elucidated (1.3,1.4) the physical application of this organism had to be optimized for limiting factors not directly nutritional. Such parameters were often decided by the engineering processes to be used (1.7). Air was essential for leaching to take place. The degree of aeration and subsequent values of dissolved oxygen in liquid systems were rate limiting for ferrous iron and metal sulphide oxidation. During ferrous iron oxidation the consumption of oxygen was far greater by the chemical components of the process than by the associated synthesis of biomass. The rate limiting value in a culture was 0.29 ppm dissolved oxygen (Liu, Branion and Duncan, 1988). Rate limitation was observed with 0.35 ppm dissolved oxygen for pyrite oxidation (Myerson, 1981). During ferrous iron oxidation, a lower limit was reached for a dissolved oxygen level of 0.20 ppm, implying that this was a minimum value for iron oxidation by T.ferrooxidans.

Beyond 1 ppm the concentration of dissolved oxygen was not rate limiting. With the influence of pressure T.ferrooxidans could grow under hydrostatic pressures of between 25 and 30 MPa but hyperbaric oxygenation was inhibitory for oxygen concentrations between 25 and 49 ppm (Davidson, Torma, Brierley and Brierley, 1981). To avoid limitation adequate engineering was required to optimize gas diffusion rates. With agitated systems, this was complicated by the effect of pulp density. Mineral leaching rate decreased when the concentration of suspended solids was too high, suggested as either attrition of the cells or limitation of gas (oxygen) transfer (Torma, Waldan and Branion, 1970). Inert solids, in the form of glass beads of 63 µm size inhibited iron oxidation in shake flasks at 1% w/v but not at 15% w/v in stirred tanks (DiSpirito, Dugan and Tuovinen, 1981; Liu et al. 1988). No oxidation occurred in shake flasks with 5%

w/v beads. This implied attrition due to the gyratory action within shake flasks. In leaching pyrite from coal, a 20% w/w suspension of coal was not found to be inhibitory by means of abrasion. The diffusion of carbon dioxide was though rate limiting in the absence of additional gas sparging (Andrews, Darcoch and Hansson, 1988). The percentage of metal extraction also decreased with increasing pulp density, for pyrite (Guay, Ghosh and Torma, 1989). With chalcopyrite in sparged shake flasks the optimum extraction rate was found to be with a pulp density of 22% w/v. Extraction against pulp density was only linear up to 10% solids (Sakaguchi, Torma and Silver, 1976). With pyrite and percentage solids ranging from 1.7% to 26.3%, the yield of total iron dissolved was inversely proportional whilst rate of dissolution was directly proportional over time to percentage solids (Guay et al, 1989).

1.5.2 Iron and sulphur precipitates

The degree of leaching was affected by the available reaction surfaces. Metal extraction as a percentage, increased with decreasing particle size, even in sterile control leaches (Dave and Mathur, 1987). Size fractions of mineral samples decreased as a result of this biodegradative action. In percolating systems, such as leach dumps (1.7) this production of mineral fines caused blockage of liquid and gas flow, with drops in efficiency due to impermeability (Bruynesteyn and Duncan, 1977). Availability of reactive surfaces was often a function of pH. Values of pH in a recycled aqueous system, through a body of broken ore were determined by a balance between acidity formed on oxidation (1.1) and acid consumed by the gangue material. Pre-testing of acid consumption reduced both wastage and iron precipitation. Most ferric iron in leach systems precipitated above pH2. Precipitates and associated leach products coated mineral surfaces forming passivation layers. In shake flask leaching of chalcopyrite at 22% w/v pulp density a final copper concentration of 35g per litre was achieved. Most of the associated iron precipitated, as jarosites (eg $KFe_3(SO_4)_2(OH)_6$). Precipitation influenced the redox

potential which affected both growth and indirect leaching (Dutrizac and MacDonald,1974). The jarosite coated the mineral and copper extraction initially ceased at 60% copper extracted. This increased to 80% on regrinding and resuspending the partially leached ore (Sakaguchi, Silver and Torma, 1976). With percolation column tests using low grade porphyry ore, leaching of copper decreased after one year in operation. Even with a liquid in-flow at pH2 this was not acid enough to maintain ferric iron as a soluble ion because of gangue consumption of acid. Flocculated iron oxides and hydroxides and co-precipitates of copper occluded the mineral surfaces. The precipitates were removed by acidifying the in-flow liquor to pH 1.5. T.ferrooxidans was though inhibited at this pH and leaching had to be resumed at pH2 (Madgwick and Ralph, 1981). During a leach of chalcopyrite the removal of similar sulphur layers by solvent washing and mineral resuspension increased percentage leaching, greater than that achieved with re-grinding (Thakur, Saraj and Gupta, 1983).

1.5.3 pH

Rises in pH inhibited T.ferrooxidans. Nickel leaching was observed to cease at above pH4 and chalcocite oxidation ceased at pH 4.6-4.7 (Bosecker,1977;Back,1977). Limitation by pH also occurred with reduced sulphur substrates. In batch culture the lower limit for growth on tetrathionate and thiosulphate was pH 3.5, but this was reduced to pH 2.5 during continuous culture (Eccleston and Kelly, 1978). The toxicity was due to degradation of these sulphur compounds, unstable in acid pH (1.4.1). Toxicity and pH relationships also existed in metal inhibitions (1.4.2). The rate of arsenopyrite leaching depended on a balance between cell tolerance to arsenic and the concentration of arsenic in solution. With a controlled pH between pH 2.3-2.5 half of the arsenic leached, precipitated (Groudeva and Groudev, 1984). As the resistance to arsenic was 9g per litre in solution, growth occurred at 15-20% w/v solids for this mineral ie concentrations that generated less than that concentration of arsenic. In comparative tests on engineering processes

used to leach pyrite, growth always became limited as the pH fell, during oxidation, to pH1. Resuspension of the mineral at pH 2 allowed leaching to resume at the previous rates (Atkins, Pooley and Townsley, 1986).

1.5.4 Temperature

The rates of iron oxidation and metal leaching increased as the temperature increased. Temperature increase during the interaction of ferric iron with mineral sulphides gave a kinetic rise in reaction rate. Biologically, this was balanced by the increase in thermal death of the microbial cells as the temperature increased. When this rate of death exceeded the parallel increases in the extraction rate the optimal maximum for bacterial leaching was surpassed (Nato and Torma, 1985). Individual temperature optima varied with specific strain but generally leaching was greater at 35°C rather than at 30°C or 40°C. Pyrite oxidation was exothermic, the released heat increasing localized temperature above this optimum, especially in dump and heap leaches. Control of this phenomenon was required to optimize leaching studies and practical applications.

Environmentally, all the above factors dictated the presence or absence of acid drainage. Oxidation required low pH (1.5.3) but the limit for the presence of viable cells in drainage from coal spoil and in the spoil itself was pH 7.2. Where the spoil had lowered buffering capacity, the value of pH fell and cell numbers increased. This was suggested as evidence of acidic microenvironments within the spoil, which generated acid drainage in conjunction with the bacteria. External temperature and water flow influenced cell numbers but no cells were found in the outermost layer (1 cm) at any time, irrespective of pH (Twardowska, 1987).

1.6 ENGINEERING ASPECTS OF MINERAL LEACHING

Sites of acid mine drainage were sometimes amenable to recovery of dissolved metal values. Historically, the

bulk of metal obtained was copper and latterly uranium. The investigation of mineral dissolution revealed the possible application of biological leaching in hydrometallurgical processing. Oxidation of sulphide in the mineral to sulphate reduced the potential for pollution present in flue gas discharges from smelters. Bacterial leaching was subsequently studied experimentally on a lab-scale in order to understand the controlling parameters of the process. Observation was also made concurrently of actual leach operations. Lab scale experiments were used to investigate this leaching, optimising the parameters for given minerals and reactions. Commercial application, which was not controlled (1.6.2) was simulated in comparison (Brierley,C.L,1978; Ralph, 1985; Olson and Kelly, 1986).

1.6.1 Leaching in enclosed vessels

Uncontrolled leaching in large heaps and dumps was simulated in percolation systems. Small percolators or larger scale ore filled columns were found to be limited by gas transfer (Bryner, Beck, Davis and Wilson, 1954). Percolation transferred nutrients and oxygen, but the oxygen content could be exhausted by oxidative reactions or be limited by flooding of the ore. The liquid from percolation systems was recycled as a source of acid, ferric iron and bacteria. Replacement of oxygen and nutrients at this stage increased yields. Forced aeration at the base of columns to form air-lift percolators also increased oxygenation (Bosecker, 1984). The more controlled the leaching environment the more efficient was the oxidation by the bacteria. Reactors, designed as a result of the physical variables associated with leaching (1.5) allowed better regulation of these process variables. These leaching parameters were controllable in stirred tanks or fermenters. The gas transfer was greater than in percolation columns and this was true in all agitated systems whether shaken, impeller driven or agitated by air-flow. Agitation increased leaching rates but the dense mineral substrates were not easily kept in an homogeneous mix. Reactor design had to account for both abrasion and acid attack on the impellers and the

reactor vessels themselves (Ralph, 1985). The use of finely ground minerals in agitated reactors and controlled temperatures also increased the efficiency and rates of leaching.

Shake flasks in agitator-incubators were routinely used as a convenient method of testing batch cultures, rapidly and with multiple experimental variations (Bosecker, 1984). Flasks achieved faster rates than percolation columns and required short contact times with the mineral, in comparison. Comparative investigations were made of leaching columns, shake flasks and (air-agitated) reactors (Puhakka and Tuovinen, 1986; Atkins, Pooley and Townsley, 1986). Air agitation provided two advantages over stirred tank reactors. Firstly, the use of air maximized gas diffusion and reduced capital and operating costs associated with impeller mixing. Secondly, air-mixing could be achieved in pachuca reactors already available in the mineral processing industry. Central tubes within the pachuca column maintained more homogeneous mixing and diffusion than in simple airlift columns. A separate study, however, found greater efficiency and lower energy requirements during bioleaching of a copper mineral in a stirred tank, than in a pachuca (Acavedo, Cacciuttolo and Gentina, 1988). Complete leaching and limitation by pH was observed in all the tested systems, with a range of minerals. Efficiency and rates of leaching were generally slower in percolation columns at higher pulp density. This was often due to pH fluctuation, partially alleviated by using free-draining and not flooded columns.

Tank and vat leaching were proposed for commercial applications (McElroy and Bruynsteyn, 1978; Torma, 1978). Operation was suggested as a continuous process with recovery of copper from a concentrate or the upgrading of a lead ore via removal of contaminating metal sulphides by leaching. Economic evaluation of these and other proposals predicted viable commercial application subject to a series of environmental, economic and physical conditions prevailing. This also applied to the beneficiation of inorganic sulphur bearing coals by

bacterial leaching.

Removal of pyritic sulphur from coal was more efficient with smaller coal particle size. Commercially, small size grinding was prohibitive (Monticello and Finnerty, 1985). Coal cleaning was possible, chemically, using ferric iron as an oxidant. The bacterial regeneration of ferric iron would have reduced potential reagent costs in such a process. The cost of acid resistant reactors in coal cleaning was dependent on reaction rates. Slow reactions involved large residence times and larger volume vessels. To be cost effective, leaching rate in reactors had to be increased (Kawatra, Eisele and Bagley, 1989). Rate increases could depend on more exact optimization of plant equipment, the addition of secondary catalysts (such as silver,1.4.2), adaptation of bacterial strains, both to the substrate and any contained inhibitory minerals and the use of separate reactors to grow inocula.

1.6.2 Dump and in-situ leaching

Leaching was observed in waste piles of low-grade ore bearing rock. Bacteria were observed in effluent solutions from commercial copper leaches (Karavaiko, Kuznetsov and Golonizik, 1977). This was deliberately encouraged in dump and heap leaching concomitant to the investigations of the mechanisms involved. Bulk volume run of mine ore was dumped after a minimum of basic physical separation processes. After mining, the crushing and grinding of the rock produced heterogeneous products both in size and composition. (Flotation products were either concentrates, more amenable to reactor leaching (1.6.1) or process tailings, usually disposed of in dams and de-watered). Recovery of the metal values was referred to as solution mining irrespective of the use of variously sized dumps, mined ore underground, and in-place fractured ore bodies during in-situ leaching (Ralph, 1985; Lundgren and Silver, 1980; Brierley and Brierley, 1986).

Dump leaching of submarginal ore could be applied to large tonnages of material (up to 4×10^9 tonnes, Brierley,C.L, 1978). Operation was relatively slow and inefficient. The occurrence of sub-optimal physico-chemical conditions

were compounded in that the original engineering work involved in constructing the dumps did not take into account the requirements and limitations of bacterial leaching. The largest dumps were 1000 ft (300 m) high. Air penetration was limited to 200 ft (60 m) from the side of a dump. This and the possible occurrence of sulphate reduction and anaerobiosis below a depth of 150 ft (45 m) limited the optimal construction sizes. The physical construction did not always allow adequate percolation needs and surface compaction zones to a depth of 15 ft (4.5 m) had either to be removed or perforated prior to operation (Brierley and Brierley, 1986). Drill holes within the dump were also employed in forced aeration. Water percolating through the dumps was collected (assuming construction was on impermeable bed rock) and treated for dissolved metal values. The stripped liquor, containing bacteria was recycled. This acid liquid was distributed by drainage channels, flooding the dump, by vertical pipes or by spraying. Flooding of the ore was associated with oxygen limitation, as observed in percolation columns (1.6.1). Spraying and sprinkling aerated the spent liquor used in irrigation but increased the risk of evaporation from the dump. Make up water and adjustments to pH were necessary to counteract this. Nutrient limitations, mainly of phosphate and ammonia could also be adjusted if necessary. The recycling of leach liquor was on a continuous or non-continuous basis. Rate-limiting factors were associated with the diffusion characteristics of the gangue, influencing pH and the redox potential. Final metal recovery benefitted from pre-conditioning and wetting of the ore. For recovery of copper from sulphide a minimum of six months good aeration was required (Lundgren and Malouf, 1983).

Temperatures in leach dump environments of 60°C to 80°C were observed. Initially the activity of bacteria under these conditions was called into question (Beck, 1967). Temperatures in abandoned waste dumps, not undergoing commercial leaching were also recorded above 50°C (Harries and Ritchie, 1983). In a test leach of 200 tons of low grade copper ore in an insulated tank, hot-spots of 59°C

and more were measured (Murr and Brierley, 1978). The cell numbers of T.ferrooxidans were related to the temperature profile. High temperatures resulted in declines in the cell population which had to be above 10^5 cells per g for adequate leaching. Cell numbers were restored to maximal levels after a flush of re-cycled liquor reduced the temperature below 40°C. Investigation of areas of thermal leaching provided isolates of a thermophilic nature, contradicting the supposed unique ability of T.ferrooxidans for mineral leaching (1.8.2).

Controlled leaching (as observed in reactors) was developed with heap leaching. Restricted in size to below 10^5 tonnes, construction was based upon prepared surfaces or impermeable pads. Low grade mixed sulphide ores underwent more processing prior to leaching than material used in dump leaching. Ore particle size was generally more homogeneous and less than 10 cm. Processing the ore also concentrated it, removing much of the gangue material and it's limitations. More rapid and complete leaching was achieved with finger heaps, by limiting physical size. Reducing the height of heaps to 35 to 60 ft (10-16 m) but allowing length of hundreds of feet increased the surface to volume ratio. This both increased air percolation and reduced temperature build-up (Robinson, 1972; Ralph, 1985).

Dump and heap leaching imposed financial costs through ore processing and transportation to suitable geographical sites. Solubilization of metals including uranium occurred at the sites of mineralization. The naturally acid water could be used as a chemical leachant. Both the mine water and mined ore from stopes in a uranium mine were found to contain bacteria (Wadden and Gallant, 1985). These produced iron and acid from oxidation of pyrite and pyrrhotite in the ore. Pressure leaching of old stopes and washing of broken ore in completed stopes with the mine water increased uranium recovery. Limitations in leaching were the same as those above ground. Run of mine ore without grinding or crushing was tested in underground rock stores. Long retention times, often greater than one year were required. The leaching rate depended on ore

size and acid and iron concentrations. Oxygen was limited during flood leaching. Heap dumps (73,000 tonnes each) were constructed and trickle leached, but with periods of free draining, during which air was introduced through imbedded pipes (Wadden and Gallant, 1985).

In conjunction with in-place leaching, above, in situ leaching was also considered. This required fracturing of ore bodies in order to allow leach cycling, but without actual mining of the ore. Use of solution mining was also proposed as possible in reaching deep ore bodies (Bhappu, 1982).

1.6.3 Iron oxidation and indirect mineral leaching

Ferric iron used as an oxidizing agent became reduced to ferrous iron. Bacterial oxidation of ferrous iron in recycling the oxidant occurred in acid solution in the absence of mineral (1.1). The physiology, biochemistry and kinetics of ferrous iron oxidation were studied (1.7). The physico-chemical parameters important in mineral oxidation were not significantly different in iron oxidation.

During growth studies the appearance of cell growth on reactor walls was observed (MacDonald and Clark, 1970). Such bacterial films reduced the retention times required in vessels during continuous iron oxidation (Mehta and LeRoux, 1974). The occurrence of cell washout was reduced as the activity of the bacterial films became more important than the activity of the cells in solution during oxidation.

Increased biomass in a reactor both increased iron oxidation rate and reduced the necessary process volume for ferric iron production. Oxidation could be used either in producing ferric iron as an oxidant or in treating acid mine drainage, which contained a major proportion of dissolved iron in the ferrous form. Oxidized, this iron was precipitated during neutralization at the site of pollution. Rotating discs through a holding tank of natural acid mine water allowed the build up of bacteria on the exposed surfaces. The affinity of the organisms was dependent on mass transfer through the

layers on the discs (Wichlacz and Olem, 1983). Alternatively, the cells,associated with precipitates on the discs, could be collected from the overflow from continuous iron oxidation. Recycling of such precipitates reduced oxidation time, but precipitation of oxidised iron did not occur in natural mine water at pH 1.6. Cell recycling was used on a commercial scale, on mine drainage at pH 1.6, by using diatomaceous earth as a cell carrier. The carrier was acid stable and cell recycling allowed treatment of $5m^3$ per min of water with up to 800 ppm ferrous iron. Oxidation of 95% ferrous iron was sustained even at temperatures below 2°C. Combined precipitations and neutralisation further reduced the pollution burden on the environment in a cost effective process (Ishikawa, Murayama, Kawahara and Imaizumi, 1983).

Oxidation of ferrous iron by bacteria trapped in iron precipitates was used in commercial leaching of uranium. Pyrite ore associated with the uranium was used to provide sulphuric acid for the solubilization process. Combined bacterial leaching required finer milling of the ore than was necessary for chemical treatment. This was not cost effective, particularly as uranium extraction was also incomplete (cf in-place uranium leaching in stopes, 1.6.2). In recycling the iron used as the chemical oxidant, bacterial oxidation however, cut the costs of chemical, permanganate oxidation. Continuous additions of fresh ferrous iron to the oxidation tank reduced the lag phase during oxidation. Prior to implementation pilot tests were carried out on various process designs (Livesey-Goldblatt, Tunley and Nagy, 1977). Oxidation increased in each vessel as film deposition increased. Films formed from iron based jarosite precipitates were porous, trapping bacteria within them. Comparison of the systems was at 34°C and pH 1.75 during continuous oxidation, after film deposition had occurred. Increasing the available surface area allowed increases in the flow rate but without affecting maximum specific oxidation per unit of surface area. Differences in the vessels were controlled by oxygen concentration. This 'Bacfox' process was efficient at producing ferric iron. At high unit

production, however, ferrous iron remained in solution. Reducing residual ferrous iron concentration required reduced flow rates. With 4.4 g per litre ferrous iron input, 0.5 g per litre remained after oxidation of 8.14 g iron per square metre per hour (Livesey-Goldblatt et al. 1977).

1.7 INORGANIC OXIDATIONS BY T.ferrooxidans

T.ferrooxidans was studied in it's role in forming acid mine drainage. The chemistry involved and the application of the basic process were also investigated. The use of iron as a chemical oxidant (1.6.3), coincided with physiological studies on iron and inorganic oxidations by T.ferrooxidans. Biochemical study was made of the metabolism of the organism, incorporating the rate limiting controls in the process and the genetic composition and possible manipulation of the cells.

1.7.1 Kinetics

Rate and degree of oxidation were controlled by nutritional and other physico-chemical parameters. Values of rates were obtained for cell systems, using both oxidizable metal and sulphur species. Measurements of rate coincided with determination of saturation coefficients of cells for the individual energy species. Limitations to the oxidation of iron were also dependant both on the concentration of the ferrous iron substrate and the ferric iron product. Growth rate and oxidative efficiency were linked to gas transfer, but inhibition was observed where this was not a factor. Batch culture on ferrous iron resulted in exponential growth. Growth was not limited by carbon dioxide concentration, but the rate of oxidation was linear in it's absence. Oxidation of iron was therefore possible in the absence of growth. Ferric iron inhibited ferrous iron oxidation irrespective of the growth status of the cells, and did not affect carbon dioxide fixation. Ferric iron product inhibition of the rate of ferrous oxidation was the principal limiting factor during batch culture. The form of

inhibition was competitive. The substrate saturation coefficient, K_s (equivalent to an apparent K_m) was lowered by the inhibitor (ie value increased) but without altering the maximum oxidation rate, V_{max}. Inhibition was partially alleviated if the potassium concentration was sufficiently high. Typical batch growth rate and K_s values were 0.143 per h and 36 mM ferrous iron. Affinity for iron, as measured by K_s was greater when measured in continuous flow systems and when measured for non-growing cell suspensions. Specific data on growth kinetics and the limits of growth were provided by continuous systems. Continuous growth with iron as limiting substrate was also influenced by a non-competitive product inhibition. Cell washout was dependent on the input concentration of ferrous iron, but this occurred prematurely as a consequence of product inhibition. The dilution rate (D) could be increased for lower substrate concentrations, with a maximum of 1.33 per h measured for incomplete oxidation of 25 mM ferrous iron input. Measurement at pH 1.6 avoided ferric precipitations but iron oxidation was subject to non-competitive inhibition by H^+ ions. Substrate concentration was also inhibitory at 700 mM ferrous iron. Carbon dioxide fixation, dependant on iron oxidation was depressed in the presence of 500 mM ferrous iron. Oxidation rate was depressed by ferric iron, in all batch cultures and oxidising systems using cell suspensions, dependant on the ferrous to ferric ratio. Values of K_s measured in continuous systems of 0.7 mM to 2.4 mM ferrous iron agreed with observations of affinity in cell suspensions of approximately 0.7 mM ferrous iron (determined for a ferrous iron substrate range of 0.4 to 43 mM). During oxidation by cell suspensions, acceleration phases prior to maximum observed rate were of one to five minute duration, increasing with the concentration of ferric iron initially present. All the above measurements and observations were made with the same strain, at 30°C (Kelly, Eccleston and Jones, 1977; Kelly and Jones, 1978; Jones and Kelly, 1983; Jones, Kelly and Wood, 1985). Alternative measurement of apparent K_m for T.ferrooxidans gave values of 1.51 mM and 1.34 mM

respectively (DiSpirito and Tuovinen, 1982; Norris, Barr and Hinson, 1988; discussed in 4.6).

Differences in kinetics for various strains in continuous culture were suggested as indicative both of culture conditions used as well as inherent differences in the isolates (Braddock, Luong and Brown,1984). Maximum growth rate of 0.070 per h was calculated for an isolate from mine drainage,cultured at 22.5°C. The equivalent to K_s was 0.78 mM. The K_s for attached organisms in a pilot rotating biological contactor at 10°C averaged at 52 ppm (0.93 mM; Wichlacz and Olem, 1985). In both systems oxidation was described by a substrate function in which oxidation rate was proportional to flow rate. The mechanism of attached cell growth and iron oxidation was an interaction between growth rate and biomass concentration. The effect of product inhibition, above, was relevant to this type of system, used in ferric iron (re) generation (cf 1.6.3).

T.ferrooxidans oxidized uranium (IV), coupled with carbon dioxide fixation (DiSpirito and Tuovinen, 1982). The uranous ion was a competitive inhibitor of ferrous iron oxidation, below 2.5 mM U (IV). Co-oxidation beyond this concentration revealed complex kinetics. During strain comparison, no link was observed between apparent K_m for ferrous iron and resistance to uranium. However, the K_m for uranium (IV) of 0.128 mM was significantly lower at 0.031 mM in a resistant strain (DiSpirito and Tuovinen, 1982 a).

Substrate inhibition was not observed with tetrathionate, at the optimum growth pH of 3. The oxidation rate was higher at lower pH but apparent K_m was lower at higher pH. The affinity for tetrathionate was of the order of 0.13 mM to 8.33 mM (Eccleston and Kelly, 1978). Continuous growth, limited by tetrathionate at pH 2.5 was probably a physiological compromise by the organism. Tetrathionate grown cells could oxidize and grow on both ferrous iron and thiosulphate. Growth on thiosulphate at pH 2.5 in continuous culture was effective as thiosulphate concentration was always low due to rapid oxidation.

Apparent K_s for thiosulphate was 1.2 mM to 25 mM. Cell yields on either sulphur compound were increased by carbon dioxide enrichment (Eccleston and Kelly, 1978). Washout of tetrathionate cells occurred at D of 0.08 per h with 10 mM substrate.

1.7.2 Biochemistry

Biochemical understanding of T.ferrooxidans came from physiological data during continuous and controlled culture, and from observations of the effect of inhibitors. Energy was derived from substrates by electron transfer to a terminal electron acceptor, oxygen. Growth yield data indicated that nineteen ferrous iron ions were oxidised per molecule of carbon dioxide fixed (Kelly et al, 1977). Metabolically, sulphur was both oxidised and assimilated. The sulphate assimilation into cellular material measured during iron oxidation was depressed by 80% during oxidation of 10 mM tetrathionate (Tuovinen, 1977). Cultures were transferable from iron to sulphur substrates (and vice versa) but with reductions in the observed oxidation rates, prior to adaptation. Biochemically, the enzymes of sulphur oxidation were largely constitutive, but induction of specific proteins was caused by introducing ferrous iron substrate. The presence of sulphur simultaneously reduced the iron oxidation rate.

Cell enzymes and the components of the respiratory electron chain were studied. Studies of the cell wall confirmed it's gram negative form. Oxidation of ferrous iron occurred at the outer membranes, without the iron entering the cell (Ingledew, 1982). The internal pH of the cell was close to neutrality (Ingledew, Cox and Halling, 1977). A proton motive force created by the pH gradient coupled with the electron transfer drove ATP synthesis. Solubility of the ferric iron product influenced the redox potential (and potential energy) of the ferrous/ferric couple, which drove this synthesis. Potential energy increased with pH value, but so did the rate of ferrous iron auto-oxidation. Obligate acidophilic physiology was therefore a compromise between the energy

available and the availability of oxidizable substrate. Energy yields from ferrous oxidation were small. This was calculated as 1 molecule of carbon assimilated requiring 22.4 molecules iron oxidized (cf with the estimation from growth studies, above).

High concentrations of electron transport components allowed the rapid rates required for substrate turnover (Ingledew, 1982). Optical spectra of cell preparations revealed a range of cytochrome species including a small concentration of b-type cytochrome. The respiratory chain was blocked by oxidase inhibitors, azide, cyanide and carbon monoxide. Oxidase activity was solely ascribed to a cytochrome a_1 species. The types of respiratory chain component were similar to those observed in other bacterial systems. These included a, b and c-type cytochromes, ferredoxin, quinones, iron-sulphur centres and copper containing enzymes. Their concentration and organization (not completely resolved) was unique.

Rusticyanin was induced on transfer of cultures from sulphur to ferrous iron. This copper containing protein was soluble and stable at pH2. Blue in colour, this protein was bleached on reduction by ferrous iron (absorbance at 590 nm). Electron transfer rates were too slow for it to be the primary electron acceptor, though it was rate limiting (Cobley and Haddock, 1975; Cox and Boxer, 1978). Primary electron acceptance was proposed for iron-sulphur centres. One iron-sulphur centre was associated with a glycoprotein. Inhibition of protein glycosylation by 2-deoxy-D-glucose prevented ferrous iron oxidation, but only after transfer from sulphur medium. Growth on sulphur was not affected, nor growth on iron where glycosylation had previously occurred (Mjoli and Kulpa, 1988).

The presence of ferric iron was suggested as a prerequisite for bacterial oxidation of the sulphide moiety of copper ores. Cupric ions in solution (100 mM) prevented growth on sulphur. This was increasingly alleviated by the presence of ferric iron, up to 18 mM (Sugio, Wada, Mizunashi, Imai and Tano, 1986). Iron

oxidation was not influenced by the copper. Cobalt did not affect iron oxidation, but complete inhibition of sulphur oxidation by 1 mM Co was not prevented by ferric iron. In contrast, the evidence of similar aerobic oxidation rates of sulphur in the presence and absence of ferric iron were presented as evidence that ferric iron reduction was not obligatory to elemental sulphur oxidation (Corbett and Ingledew, 1987).

Biochemical understanding of the oxidations particularly the rate limiting steps and sites of inhibition were sought for possible future organism alteration, particularly via genetic manipulation.

1.7.3 Genetics

Bacterial physiology was subject to the genetic composition of the cell (Nicolaidis, 1987). Selection of both natural and induced mutants resulted in enhanced oxidation rates. T.ferrooxidans was sensitive to and mutated by UV radiation, ethylenimine and nitrosoguanidine (Grudeva, Markov and Gentchev, 1980). Mutated strains maintained greater oxidation rates than wild parent strains when used in lab scale apparatus similar to the Bacfox process (1.6.3; Groudev, 1981).

Natural and induced selection changed the genetic characteristics, but did not introduce new characteristics. Application of recombinant DNA technology was required. Naturally occurring plasmids had no apparent function in iron oxidation and were cryptic (Martin, Dugan and Tuovinen, 1981) with one exception indicating conferred resistance to uraninite. Metal tolerance was a useful marker for cell selection. Gene replication and expression in E.coli was the preferred tool for study of Thiobacillus genetics. Work concentrated on the possibility of eventually expressing recombinant genes in T.ferrooxidans. The aim was to construct plasmids capable of improving resistance to metals and promoting iron oxidation (Nicolaidis, 1987). To this end plasmid replication in E.coli and the cloning and expression of the glutamine synthetase gene were successful (Rawlings, Pretorius and Woods, 1984; Barros,

Rawlings and Woods, 1985).

Phenotypic variations occurred in structural morphology. The expected polar flagella were replaced by peritrichous flagella in some strains and were absent in any form in others. Variability in distribution of pili was also present (DiSpirito, Silver, Voss and Tuovinen, 1982). Direct studies of a variable range of T.ferrooxidans cells resolved the strains of this one taxonomic species into seven groups related and separated by their DNA homologies (Harrison, 1982, 1984). This separated the strains at species level and at genus level given the lack of homology. One homology group was identified with a previously separately classified organism, Leptospirillum ferrooxidans (1.8).

1.8 ALTERNATIVE ACIDOPHILIC ORGANISMS

Bacterial mineral leaching studies were intially concentrated on T.ferrooxidans. These revealed physiological, morphological and genetic differences within isolated strains (1.7). Regions of heat liberation in mineral leaching dumps inhibited the cells (1.5.4). Isolations, descriptively different from the type strain also differed genetically (1.7.3). Organism types had already been separated on their use of heterotrophic metabolism (1.3.2). Some organisms, putatively thiobacilli-like were enriched from regions of thermal activity (1.8.2). All these organisms were variously described and tested relative to their role in inorganic oxidations and mineral dissolution.

1.8.1 Mesophilic bacteria

Acidophilic heterotrophs were separated from heterogeneous cultures (1.3.2). T.acidophilus oxidized sulphur but neither iron nor metal sulphide. A.cryptum also degraded elemental sulphur but only incidentally to obligate chemoorganotrophy. Both organisms grew on glucose at pH 3.5.Acidophilium was found at sites of acid coal drainage. Physiological study of these isolates extended the species

range in this genus (Wichlacz, Unz and Langworthy, 1986). Some organic material in these environments leaked from autotrophic organisms, principally T.ferrooxidans. Certain organics such as pyruvate inhibited T.ferrooxidans. Both heterotrophs could grow on the pyruvate and alleviate this inhibition (Harrison, 1984). This provided a mechanism for the enhancement of mineral leaching by heterotrophs in mixed cultures in natural leaching environments.

T.thiooxidans was isolated as an autotrophic sulphur oxidizer (Waksmann and Joffe, 1922). It could not utilize organic material nor oxidize ferrous iron. Growth on elemental sulphur greatly reduced pH to acid levels of pH 1.5. Cultures were also possible on thiosulphate media. Morphology differed within strains and as with the genomic differences in T.ferrooxidans, species diversity was suspected (Harrison, 1982). Incapable of oxidizing metal sulphide, T.thiooxidans could degrade sulphur based deposition layers formed on mineral surfaces during dissolution. This organism was present in many heterogeneous enrichment cultures. Environmentally, the rapid oxidation of sulphur may have altered the environmental growth conditions in favour of T.ferrooxidans. Natural mixed cultures degraded pyrite more rapidly in comparative studies than pure cultures. Mixed cultures of isolated strains of T.ferrooxidans and T.thiooxidans did not match this degree of dissolution. Similar degrees of dissolution were obtained with mixed cultures of T.thiooxidans and Leptospirillum ferrooxidans (Norris and Kelly, 1978a).

L.ferrooxidans differed morphologically, physiologically and genetically from T.ferrooxidans. Isolated from a copper leach site, this mesophilic acidophile formed long spirals from individual vibrios. These were capable of autotrophic iron oxidation, but not sulphur oxidation (Markosyan, 1972; Balashova, Vedenina, Markosyan and Zavarzin, 1974). This mobile chemolithotroph was structurally gram-negative. The cell, which formed

gelatinous sheaths, displayed a high degree of polymorphism during growth (Pivovarova, Markosyan and Karavaiko, 1981). A combination of sheath formation and filamentous forms may have caused the cell aggregation observed in batch ferrous iron culture. This was associated with sub-optimal growth temperature, occurring at 25°C but not 37°C (Harrison and Norris, 1985).

Growth was not observed with organic compounds and neither yeast extract nor glucose influenced ferrous iron oxidation (Eccleston, Kelly and Wood, 1985). Initially growth was not observed with mineral sulphide. Mixed cultures of L.ferrooxidans and T.organoparus (identical to T.acidophilus) allowed complete dissolution of both pyrite and chalcopyrite (Balashova, et al, 1974). This ability to oxidize minerals in the complementary company of a sulphur oxidizer was also observed with T.thiooxidans.

Subsequently, L.ferrooxidans was found capable of autotrophic pyrite oxidation in pure culture. Enrichment cultures on pyrite from a diverse range of mineral leaching sources, provided isolations of Leptospirillum-like bacteria also capable of pyrite oxidation (Norris, 1983). These isolates were distinct from T.ferrooxidans based on electrophoretic gel protein patterns. Differences were also apparent between the isolates. Based on DNA-DNA homologies, at least two separate genomic groups of Leptospirillum-like bacteria (including L.ferrooxidans were distinguished (Harrison and Norris, 1985).

In batch ferrous iron culture, L.ferrooxidans had a 10.5 h doubling time compared to 5.5 h for T.ferrooxidans (Eccleston, et al, 1985). Ferrous iron substrate was inhibitory to cell suspensions, preventing oxidation at 800 mM concentration. Kinetically, ferric iron competitively inhibited iron oxidation, but the estimate of K_i of 33 $\pm$ 13 mM ferric iron was significantly higher than for T.ferrooxidans (1.7.1). Apparent K_m values for ferrous iron oxidation were averaged at 0.5 $\pm$ 0.3 mM ferrous iron. Additional estimations placed the apparent K_m at 0.25 mM (Norris et al. 1988; 4.2.3). Cell

suspensions lost activity over time, associated with cell clumping and lysis, but no effect on oxidation rate was caused by a pH range of pH 1 to pH 2.

Batch pyrite cultures of T.ferrooxidans were limited by pH (1.5.3), but natural mixed cultures extended dissolution beyond this point (Norris 1983). Growth at 30°C of L.ferrooxidans on pyrite was also more extensive. Certain of the Leptospirillum-like bacteria oxidized pyrite at an initial pH of pH 1.3, inhibitory to T.ferrooxidans. The degree of dissolution was similar to natural mixed cultures indicating that this extension of dissolution was due to Leptospirillum-like bacteria in these cultures. Domination of Leptospirillum cells was observed in mixed cultures after continuous sub-culture. However, comparing pure cultures, 87% of Leptospirillum cells were estimated as being associated with mineral during dissolution compared to 7% for T.ferrooxidans. In mixed culture approximately 38% of cells were attached to the mineral (Norris et al, 1988).

During continuous pyrite oxidation in an airlift reactor, Leptospirillum-like bacteria displaced Thiobacillus cells at pH 1.5. The Leptospirillum numbers dropped almost to zero at pH 2.3 and had no obvious influence on leaching during discontinuous operation (Halls and Onken, 1988). The drop in pH during pyrite oxidation was greater with T.ferrooxidans than L.ferrooxidans, except when T.thiooxidans was also present, but this did not influence the rate or degree of metal dissolution. Sulphur oxidation in mixed cultures was dependent on a high degree of iron oxidation (70%) occurring first, at higher (5% w/v) pulp density (Norris, 1983).

The influence of iron oxidation kinetics, particularly the relative resistance to ferric iron may also have been selective for Leptospirillum cells as well as pH. Leptospirillum was also more sensitive to copper than T.ferrooxidans, though via a different form of inhibition (Norris et al, 1988; 4.2.6). However, enrichment cultures with Leptospirillum-like cells were abundant with 25 g per litre Cu present (Harrison and Norris, 1985).

1.8.2 Moderately thermophilic bacteria

Mineral leaching habitats contained a heterogeneous population of acidophilic organisms, some limited by temperature. Thermal springs and sites of oxidation provided a range of thermophilic isolates of Thiobacillus type physiology.

Isolate TH1 from a hot spring grew on 1% w/v pyrite at 50°C with a 0.02% w/v yeast extract(YE) supplement at pH 3.28. On falling to pH 1.3 this value was growth limiting. Strain TH1 was also capable of ferrous iron and thiosulphate oxidation (LeRoux, Wakerley and Hunt, 1977). Described as a chemolithotrophic heterotroph, yeast extract was not required for ferrous iron oxidation by cell suspensions. Ferric iron product at pH 1.6 was a competitive inhibitor of iron oxidation. Iron oxidation during growth at this pH gave a doubling time of 3.75 h (Brierley, Norris, Kelly and LeRoux, 1978). Measured in suspensions the apparent K_m was 7.3 mM ferrous iron and the K_i was 2.9 mM ferric iron. A much lower apparent K_m of 1.04 mM was subsequently measured (Norris et al, 1988; 4.2.4). The growth requirement for yeast extract was replaceable with 20 ppm glutathione. Less than 25% iron oxidized was possible with thiosulphate or tetrathionate (100 ppm) as the supplement. Suitably supplemented, TH1 grew on pyrite and copper sulphides. It did not grow heterotrophically or through continuous sub-culture on sulphur. Similar bacteria were isolated from commercial and test copper dump leaching operations, known to be thermally active (Brierley, 1978). These were designated TH2 and TH3. Morphologically similar, these bacterial rods were larger than those of T.ferrooxidans. Various site isolations were consequently labelled TH4, TH5 and TH6 (Brierley and Brierley, 1986). Strains TH1 and TH3 had different limiting concentrations for yeast extract on pyrite. The requirement of strain TH1 for yeast extract was resolved as a requirement for reduced sulphur and carbon. However, thiosulphate supplements and the use of carbon dioxide enriched air during culture resulted in autotrophic iron oxidation both by strain TH1 and several isolates from washed coal piles and waste coal spoil heaps

(Marsh and Norris, 1983a). Optimum growth temperatures of 50°C with 0.02% w/v YE were reduced by approximately 5°C during this autotrophic growth on ferrous iron, for all these isolates, except for strain TH1 and the designated BC1 strain. No growth of any isolate or enrichment culture was observed at 60°C hence the designation of moderate thermophile. Variations with growth conditions and substrates existed in the observed temperature ranges. The optimum pH for growth was between pH 1.5 and pH 1.8. Iron oxidation rate by strain (isolate) BC1 was not affected by the degree of carbon dioxide enrichment in the air but the rate was reduced by a factor of four in the absence of carbon dioxide enrichment (Marsh and Norris, 1983a). During pyrite oxidation, strain TH1 was limited by acidity at pH 1.2. Pyrite dissolution resumed after resuspensions at pH 2 both of strain TH1 and the Kingsbury enrichment (Marsh and Norris, 1983b). Autotrophic oxidation and release of copper was greater at 50°C with the LM enrichment and the ALV isolate. With the enrichment culture the greater degree of dissolution was marked by a slower growth rate than those observed at lower temperatures.

The moderately thermophilic acidophiles were studied physiologically to elucidate their discovered chemolithotrophic metabolism, on using carbon dioxide enrichment. Isolates designated ALV, BC and K (after sites of isolation) all grew autotrophically with ferrous iron, carbon dioxide and mineral salts provided reduced sulphur was present (Wood and Kelly, 1983). The sulphur was only used in biosynthesis. The enhancement of iron oxidation rate by yeast extract was dependent on ferrous iron oxidation, due to obligate mixotrophy. Glucose, when present, was therefore used simultaneously with carbon dioxide as a carbon source with all these isolates. No growth occurred on glucose alone (Wood and Kelly, 1983). Strain ALV was noted to assimilate sulphate during iron driven growth with 1 mM trisodium citrate (Wood and Kelly, 1984). Wood and Kelly (1983) noted that growth yield varied with the source of reduced sulphur. More detailed investigation of the source of reduced sulphur showed the

concentration to be growth limiting and with strain ALV actually unnecessary (Norris and Barr, 1985; 3.2.4). This iron oxidation with sulphate assimilation was also shown with Sulfobacillus thermosulfidooxidans. Sulfobacillus differed from the above isolates being a gram positive spore forming organism. Oxidation of iron, elemental sulphur and sulphide ore was accomplished in the presence of 0.02% w/v YE (Golovacheva and Karavaiko, 1978). Maximum leaching was at 50°C.

1.8.3 Thermophilic bacteria

An acidophilic isolate with a spherical morphology, capable of inorganic oxidation at high temperature (up to 85°C) was isolated from hot acid springs. Structurally the cell wall was not comparable with either typical gram negative or gram positive organisms. Similarities to extreme halophiles placed the isolate in the classification of archaebacteria. Designated as Sulfolobus this organism was a facultative autotroph capable of both sulphur oxidation and growth on yeast extract. Carbon dioxide enrichment of the gas phase enhanced sulphur oxidation (Brock, Brock, Bally and Weiss, 1972). A similar isolate, also designated Sulfolobus (acidocaldarius). was incapable of growth on yeast extract as an energy source, but oxidised both ferrous iron and sulphur as substrates (Brierley and Brierley, 1973). Isolates were enriched from metal leaching environments associated with self-heating and washed coal piles (Marsh and Norris, 1983a). Homology studies divided the genus Sulfolobus into separate species, S.acidocaldarius, S.brierleyi and S.solfataricus. Further isolations recovered similar bacteria capable of switching to anaerobic growth. Designated as genus Acidianus. the species S.brierleyi was re-classified in this genus (Segerer, Neuner, Kristjansson and Stetter, 1986). The strains putatively ascribed to S.acidocaldarius were capable of chemolithotrophic growth on metal sulphides and soluble reduced sulphur species.

Utility of Sulfolobus as an alternative to T.ferrooxidans was dependent on growth and oxidation in similar

environmental conditions, altered only by the optimum temperature of growth. Enhanced extraction rates could be attributed to the influence of heat on chemical release of copper from non refractory ores. With chalcopyrite Sulfolobus was capable of greater dissolution than T.ferrooxidans not attributable to temperature alone (Brierley and Brierley, 1986). Copper extraction of 90% from copper concentrates contrasted with typically incomplete leaching by T.ferrooxidans of a maximum 50% extraction. In a comparative test with S.acidocaldarius (Birch Coppice), 83% extraction at an overall rate of 11.5 mg per litre per h Cu was achieved at 68°C compared to 19% extraction at 2.5 mg per litre per h at 30°C. (The thermophilic leach had two stages the faster of which was at 36 mg per litre per h Cu). Achieved with a 20 day residence time in shake flasks, the maximum extraction rate was increased to 50 mg per litre per h in a semi-continuous air-lift reactor with 15% w/v solids. The percentage recovery overall was still greatest with the longest residence times (LeRoux and Wakerley, 1988). Dissolution rates and percentage extraction differed with the strain of Sulfolobus used (Marsh, Norris and LeRoux, 1983). Extraction of molybdenum from molybdenite was enhanced by the presence of yeast extract (Brierley and Murr, 1973), though growth was partially inhibited with 750 mg per litre Mo in solution. Using S.acidocaldarius to oxidize pyritic sulphur in coal, the presence of 0.02% w/v yeast extract reduced the soluble sulphate concentration from 14 g to 8 g per litre (Kargi and Robinson, 1982a).

This organism was studied in a range of process designs. The use of an air lift fermenter with external recycling was expected to minimize particle attrition and shear forces, whilst increasing gas transfer (Kargi and Cervoni, 1983). Parameters controlling coal desulphurization were found to be similar to those observed in metal sulphide leaching and with mesophilic organisms. Sulphur removal was controlled by the coal particle size (available surface area), sulphur content and the pulp density of operation (Kargi and Robinson, 1982 a,b). At 60°C

Sulfolobus removed 90% of the pyrite from a coal in four to six days as opposed to fifteen to twenty days at 25°C with T.ferrooxidans in comparable tests. Expected reduced capital costs for the reduced residence time in any proposed thermophilic process were offset by the increased operating costs at the higher temperature. In perspective, comparing Sulfolobus to actual T.ferrooxidans leaching operations, in the pre-oxidation of gold ores, the thermophile was regarded as a possible alternative if not superior organism to T.ferrooxidans due to it's potential to leach ores at a significantly faster rate (Lawrence and Marchant, 1988). Application would only be feasible if Sulfolobus could be adapted or the process controlled to accommodate similar pulp densities to mesophilic systems. In cost benefit analysis both capital and operating costs increased with the size of required residence time for a fixed pulp loading (30% w/v). These costs also increased as the practical value of pulp loading decreased, counteracting the reduced residence time allowed due to faster reaction kinetics.

Growth of Sulfolobus species was subject to nutrient limitation and metal toxicity. Inhibition of Sulfolobus LM (Marsh and Norris 1983b) with 3 g per litre Cu in solution was gradually alleviated by selective adaptation. Growth of strain BC was eventually noted with 27g per litre Cu in solution. Limited oxidation of arsenopyrite was also reported as being due to toxicity, of 1.5 g per litre As in solution. In all these leaches, high ferric iron concentrations and deposition of ferric sulphate complexes occurred near depletion of sulphide leaching (Norris and Parrott, 1986). Reducing the risk of precipitation in possible commerical operation was balanced by nutrient requirements. During coal desulphurization, one nutrient medium was optimal for sulphur removal of 28 mg per litre per h S for N/P and N/Mg ratios of 47.5 and 11.5 respectively. The source of the nitrogen (as various ammonium salts) at this ratio did not influence rate (Kargi and Robinson 1985).

Thermophilic iron oxidation was important in mineral dissolution, ferrous iron appearing in solution prior to

the target metal (Norris and Parrott, 1986). The affinity for iron was measured as 0.56 mM ferrous iron and ferric iron was found to be a competitive inhibitor (Norris *et al*, 1988; 4.2.5).

1.9 Project aims

The majority of process information relating to ferrous iron oxidation and mineral sulphide dissolution was developed with *T.ferrooxidans*. This organism was extensively studied physiologically, biochemically and genetically, including the composition of the electron transfer respiratory chain and the control of ferrous iron oxidation by ferric iron product inhibition. The physico-chemical limitations of pH, temperature, redox, pulp density, particle size and gas and nutrient transfer were also measured for mesophilic mineral dissolution. Commercial application concentrated on this organism. Environmentally, the influence of acidophilic mesophilic heterotrophs was predicted from laboratory studies on mixed cultures and particularly the reduced inhibition of *T.ferrooxidans* on removal of the build up of organic by-products by the heterotrophs. The influence of the range of acidophilic strains subsequently isolated was open to conjecture. Interest was concentrated largely on their capacity for mineral dissolution, particularly at higher temperatures. Physiological studies spread the abilities of various isolates over a range of reported growth conditions including the need for growth supplements, especially reduced sulphur. Other than the observation of iron oxidation the capacity of these isolates for iron oxidation was largely unmeasured. Therefore no direct comparison with *T.ferrooxidans* was available. This project therefore aimed to provide a comparative study of the ability for iron oxidation and the influences on observed growth relative to iron oxidation for a range of acidophilic iron oxidizing bacteria. (Organisms studied, strains and isolates, are recorded in 2.1). Growth conditions were altered to try and finalise the requirement both qualitatively and quantitatively for reduced sulphur. The principle was then to culture the organisms in optimum conditions in order to develop a

method of comparatively measuring the kinetic abilities of the organisms for iron oxidation and the role of ferric iron. This project therefore reports the attempt to (1) elucidate growth requirements for moderate thermophiles, (2) measure the ability of a range of acidophiles to oxidize iron, as a K_m value, (3) relate the relevance of any kinetic measurements to growth, (4) compare the isolates on a biochemical basis, (5) relate the relevance of iron oxidation to mineral dissolution.

2 MATERIALS AND METHODS

2.1 ORGANISMS

The organisms in this study were all acidophilic iron oxidizers. Except for strain TH3 all were capable of chemolithotrophic growth with ferrous iron substrate (Chapter 3). The principal division of organisms was growth temperature, incubation being routinely at 30°C for mesophilic growth, 45°C for moderately thermophilic growth and 65°C for thermophilic growth. (This was for batch growth flasks and stock cultures, fermenter vessels being more precisely incubated).

The mesophiles were designated as either Thiobacillus ferrooxidans or Leptospirillum ferrooxidans, each represented by two strains or isolates. T.ferrooxidans was either a type strain, Deutsche Sammlung von Mikroorganismen 583 or an isolate designated strain LA from a mine water sample of the uraniferous Los Amoles mine, Municipio de Rayon, Sonora, Mexico. (Unless specified in the text the strain used was DSM 583). These strains had little DNA homology, neither did the Leptospirillum strains (Harrison, 1982; Harrison and Norris, 1985 ;1.7.3,1.8.1). The Leptospirillum strains were L.ferrooxidans strain Markosyan (Markosyan, 1972 - originally supplied by G A Zavarzin) and an isolate from a coal pile at the Birch Coppice colliery, Warwickshire. This isolate, strain BC, was described as a Leptospirillum-like organism (isolation and description of Leptospirillum-like bacteria, Norris 1983).

Moderate thermophiles were isolated from enrichment cultures and designated strains TH1, TH3, BC1, LM2 and ALV. The original sample sites represented a diverse range of metal and/or sulphur bearing environments. (For description and sites of isolation see 1.8.2). Strain TH3 was originally from a copper leach dump at the Chino

Mine, New Mexico, USA (Brierley,J.A, 1978) but was not maintained in any culture collections. An isolate from a new sample of the same dump was redesignated strain TH3 having the same morphological and physiological characteristics as those originally described (Norris, personal communication; Brierley, 1978).

A thermophilic Sulfolobus-like organism was isolated from the Birch Coppice site, Warwickshire (Marsh and Norris, 1983a) and referred to as Sulfolobus strain BC65.

All the organisms were maintained as working stock cultures in shake flasks and were routinely sub-cultured. Separate stock cultures were maintained on different substrates and kept static at 4°C or at room temperature. These were sub-cultured and incubated (prior to restorage) at regular, monthly intervals.

2.2 ORGANISM GROWTH - MEDIA, CONDITIONS AND MEASUREMENT

2.2.1 Culture media

Liquid culture media were based on two different mineral salts solutions. Prepared in glass distilled water, pH of the media were adjusted with 5% v/v sulphuric acid (in distilled water). Based on nutritional requirements for T.ferrooxidans the composition of both salts solutions had been previously identified by comparative growth studies as being the most efficacious for growth of all the organisms in this study (Marsh, 1985). The salts solutions are summarized in table 2.1.

Adjustments to pH were made before sterilization and/or addition of energy source substrate. Enriched salts were used at pH 1.7 (unless specified) with ferrous iron as the energy source and 9K salts (without the ferrous iron or calcium nitrate of Silverman and Lundgren,1959) were used at pH 2 with solid mineral sulphide. The more concentrated 9K salts supplied nutrient requirements for the greater cell yields generated with mineral sulphide than generated with ferrous iron substrate and the higher initial pH was due to acid production during sulphide oxidation.

TABLE 2.1

Chemical composition of salts solutions used as the basis of all liquid culture media for the acidophilic iron oxidisers. Figures are of g per litre distilled water.

	Enriched Salts	9K Salts
$MgSO_4$	0.4	0.5
$(NH_4)_2SO_4$	0.2	3.0
K_2HPO_4	0.1	0.5
KCl	0.1	0.1

Ferrous iron stock solutions were aqueous ferrous sulphate at 278 g per litre at pH 1.3. Ground mineral sulphide was washed and sized prior to use (see appendix 1, for mineral preparation) and used at concentrations between 1% and 10% w/v. At low, 1% w/v, mineral concentration, enriched salts (ES) pH2 was used as the medium to reduce jarosite formation increased in the higher ammonium concentration of 9K salts (this was outweighed by nutritional demand at higher mineral concentrations).

Media supplements were added during investigations of physiology (Chapter 3). Summaries of supplements and conditions of use are given in table 2.2. The supplements were added separately, aseptically, after the salts solutions were sterilised in an autoclave at 15 psi for 15 min. (The mineral sulphide was autoclaved with the salts solutions). The lower sterilisation temperature generated at 10 psi prevented chemical oxidation. Ferrous iron was also prepared at pH1.3 to prevent auto-oxidation and sterilised separately from the mineral salts to avoid precipitation.

Ferric iron, during investigation on it's effect on batch growth(4.3) was supplemented as ferric sulphate. Ferric iron had a solubility in aqueous solution of little more than 1M limiting the concentration of stock solutions and of final concentrations in culture media. Stock solutions of 0.5 M ferric sulphate (281 g per litre) were used. That

TABLE 2.2

Media supplements for acidophilic iron oxidizers. Concentrations in mM for aqueous solutions sterilized in steam at x psi for y min.

Supplement	Stock Soln	Used Concentration*	Sterilization psi/min
glucose	100	1	15/15
sodium thiosulphate	100	1	10/10
potassium tetrathionate	100	1	10/10
yeast extract	1%w/v	0.01%w/v	15/15
ferrous sulphate	1000	50	10/10
mineral sulphide	-	1%w/v	15/15

* Standard concentration for batch culture of 100 ml volume

is, 12.5 ml 0.5 M hydrated $Fe_2(SO_4)_3$ 100 ml total resulting in a final concentration of 125 mM ferric iron. To maintain the overall composition of the basic enriched salts , the ferric iron was added to a salts solution with 12.5% greater salts concentration than the "recipe" in table 2.1. However, the resultant pH value was always less than pH 1.6 due to the acidity of aqueous ferric sulphate, even if the salts to which the iron was added were at pH7 to 7.5. The ferric sulphate was dissolved in 0.1M sodium hydroxide and added in a 1:8 ratio as above, to salts not adjusted for pH. This increased the ease of solubility of the ferric iron stock (still slow) and produced a consistent final pH of pH 1.8. Control flasks of enriched salts were also used at pH 1.8. This introduced significant concentrations of sodium into the experiment (62.5 mM), further constraining the maximum final ferric iron concentration used (125 mM). As a control sodium was used separately from ferric iron, as

4.44 g per litre Na_2SO_4. The ferric iron solution precipitated on autoclaving (even at 10 psi for 10 min) and was filter sterilised using 0.22μm filters prior to use. When different ferric iron concentrations were used the additions were made to 100 ml volumes, the recorded concentrations adjusted to allow for overall volume. Therefore, total additions were kept equal, with sterile water, to conserve comparable ferrous iron substrate concentrations.

2.2.2 Growth conditions - Batch culture

All glassware was prewashed in concentrated hydrochloric acid to remove any adsorbed metals from the glass. Glass shake flasks (250 ml) were used to grow stock cultures and during the measurement of growth curves. These were agitated in rotary shakers at 120 rpm with 100 ml total volume (before inoculation) irrespective of substrate. Flask necks were plugged with polystyrene foam bungs, inserted with cotton wool plugged glass tubes when a supply of carbon dioxide enriched air was required - for all the moderate thermophiles and Sulfolobus BC65 (except when supplemented with yeast extract). Bungs and rods were autoclaved individually wrapped. The mesophiles were aerated solely by diffusion. The same conditions were used for iron grown cultures in 2 l shake flasks, with 1 to 1.5 l of medium containing 50 mM ferrous iron (table 2.2). Bulk culture of iron grown cells for respiratory chain studies (Chapter 5) was in 20 l glass carboys. Each carboy was autoclaved sealed with a silicone rubber bung at 15 psi for 40 min with 17.1 l of a concentrated salts solution at pH 1.7. This medium was diluted to normal enriched salts/ferrous iron strength with 900 ml of sterile 1M ferrous sulphate solution. Aeration was a filtered supply via a glass tube passed through the rubber bung. Each carboy holding a magnetic stirrer bar was mounted above a magnetic/heater stirrer on a steel spread plate itself supported by bricks. The plates spread both the weight on the stirrers and the heated surface area in contact with the carboys. Filtered air exhausts were placed in the bungs.

Growth with mineral concentrations of greater than 1% w/v was in fermenter vessels, also used to test specific growth conditions in ferrous iron supplemented media (and for continuous culture, 2.2.3). Vessels were glass with a ground glass flange on the neck fitted (for approximately three quarters of the total volume) with a glass water jacket. Metallic components were not used in contact with any media, to avoid corrosion and subsequent metal contamination of samples due to combined effects of heat, acidity and organisms. Working capacity of the vessels was generally 70% of the total 500 ml or 1 l volumes. Temperature was maintained via the water jackets by thermostatically controlled Churchill circulating water pumps. Glass lids were fitted to the glass flange by a metal clamp with a teflon or silicone rubber seal. Each lid incorporated five Quickfit ground glass sockets for access of services. Firstly, a thermometer was placed in the medium in a sealed glass finger fitted in the lid and filled with glycerol. Secondly, a sintered glass sparger fitted to a glass cone was used for aeration. These clogged with mineral substrates at 68°C (cleaned by boiling in concentrated hydrochloric and nitric acids) and were replaced by pipettes fitted in rubber bungs, under these conditions. Before being sparged, gas and gas mixtures were filtered and when required humidified in water-filled Drechsel bottles. Thirdly, reflux condensers to counter evaporation were fitted in the lid, and were plugged with cotton wool. Liquid levels in the vessels were kept to predetermined levels with sterile water. Fourthly, mineral substrate was agitated by motor driven impeller via a vertical drive shaft fitted into a glass joint in the lid sealed with silicone rubber O-rings. Overhead electric motors were supported by clamp stands. Metal shafts were initially used coated with teflon, but the teflon coat failed at points of wear in the seal in the lid and completely glass shafts and paddles were then used. These had to be carefully set up to prevent snapping but once set were generally operated without incident. In the absence of mineral a magnetic bar was placed in the medium and the vessel was mounted on a

magnetic stirrer. Magnetic stirring was inefficient and created a grinding action on the glass with mineral solids. When using a magnetic stirrer the spare port was sealed with a ground glass stopper. Fifthly, access was required for sampling.

When required, complete vessels with fitted stirrer, condenser, Drechsel bottle and medium were autoclaved but mineral supported growth of Sulfolobus BC65 was in non sterile growth conditions. Every vessel was equilibrated to final operating conditions for several hours prior to inoculation. Inocula were 5% to 10% v/v from liquid batch cultures grown under the same initial culture conditions. Experimental set-up of vessels is demonstrated for mineral grown cultures in fig 2.1.

2.2.3 Growth conditions - Continuous culture

Vessels as in 2.2.2 were adapted for growth on ferrous iron in continuous culture. Access ports accommodated medium in- flow and oxidized medium outflow. Some vessels were used with weir type overflows either as a side arm in the vessel wall as in the Bacfox type model (4.4.2) or as a length of glass tube, passing at right angles through a silicone rubber sealed port in the vessel wall(used with L.ferrooxidans (4.4.1). Air exhaust was filtered directly or passed through a condenser first.Without a gravity overflow, air exhaust and waste medium overflow were combined. A glass tube fused to a hollow glass cone was cut to reach into the vessel to the desired volume level. On blocking all gas exits, this level was preserved by the pressure of inflowing air blowing out the excess liquid via the tube. This system was used with L.ferrooxidans (4.4.4), T.ferrooxidans (4.4.3; 4.4.4) and strain ALV (4.4.3). The alternative was to provide a separate exhaust and pump out the waste medium at a rate greater than input (4.4.1). Within this framework conditions were adapted as necessary and the experimental results include details of specific volumes and substrate concentrations used.

In investigating the principles of Bacfox operation for T.ferrooxidans (4.4.2) two straight sided vessels were

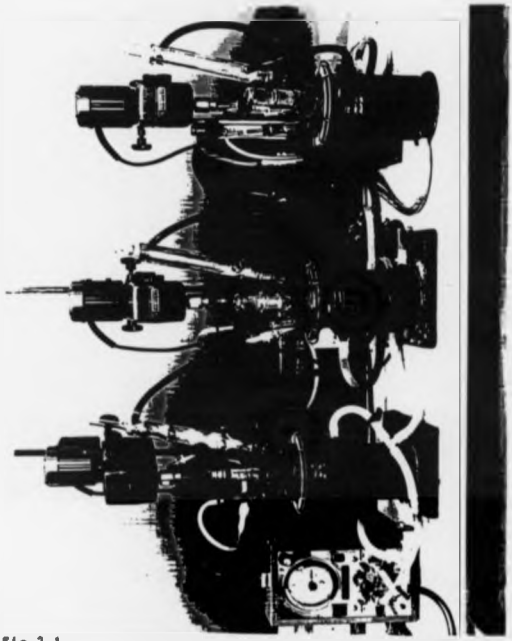


Fig 2.1
Equipment used in batch mineral leaching experiments. Centrally stirred glass vessels, fitted with water jackets, were connected to Churchill circulating water pumps operated at 71°C. Restricted flow of heating water ensured equal flow and an equilibrium of 68°C (±0.5°C) for the media in all the vessels. The vessels were insulated from the support stands by cardboard discs. The stands supported electrically powered motors, driving a glass shaft fitted vertically through the centre of each lid. The lids were sealed with flat silicone rubber gaskets. Double surface reflux condensers were connected in series when using multiple units. Sampling ports were fitted with ground glass stoppers to further reduce evaporation. The rear ports in each lid held thermometers in glycerol filled glass fingers. Pipettes placed in each vessel in rubber bungs, allowed point injection of air, filtered through glass wool after passage through flow meters. (The red-brown colouration of the glass at the top of the vessels, was ferric iron formed during the experiment).

fitted with open-ended glass tubes. The vessels were capped with petri-dish lids to reduce evaporation. Each tube was Pyrex of between 5 cm and 5.5 cm in length and external diameter of 8 mm. A disc was cut from 3 mm thick perspex (78 mm diameter) and mounted on two solid perspex legs of 1 cm height and placed in the base of the vessels. This was drilled with holes, centrally and near the edge of the disc. Allowing for both holes and legs the remaining area of the disc was drilled with further holes of ¼ inch diameter. This imperial measurement best matched the internal diameter of the glass tubes. Each hole had a glass tube fixed above it with Araldite epoxy resin, the centres having been positioned to allow for the clearance of 3mm of glass where tubes touched. These mounted tubes were placed above a magnetic stirrer bar, the vessels sitting on the stirrer. The combined internal height was below overflow level. Three access holes were cut into the lid. At the centre a pipette passed down the centre hole of the disc for aeration, at the back a thermometer sat in the medium, and at the side medium was fed in through a glass medium break fitted into the edge of the disc. Both air and fresh medium arrived at the base of the vessel, were distributed by the stirrer and fed up through each tube. (This was tested for an uninoculated vessel, each tube appearing to be individually, and equally, aerated).Estimation of added surface area was based on the surface area of a cylinder ie pi x d x h, in cm^2. For the empty vessel this was vessel diameter (80 mm) x medium height (80 mm) x 3.14 ie approximately 200 cm^2 (excluding base). With tubes, both the inside and outsides had to be accounted for with diameters of 6 mm and 8 mm respectively and average height of 5.25 cm. Each tube therefore had 24.7 cm^2 of surface area available. In the experiment, the vessel of pH 2 had 41 tubes added and that at pH 1.6, 38 tubes (4.4.2).This gave additional surface area of greater than 950 cm^2, but was probably less in practice given the surface to surface contact of glass tubes. By estimating that external surface area of tubes was reduced by 50% this gave a total available internal surface area approaching 900 cm^2, 4.5 times that available in the empty

vessel.The apparatus is diagrammatically represented in fig 2.2.

Fresh medium for continuous iron oxidation was kept in sterile 20 l glass carboys prepared as in 2.2.2, including stirring in order to keep the ferrous iron in an homogeneous mix. These reservoirs of enriched salts were of pH values and with concentrations of ferrous iron dependent on the requirements of the experiment.Media was pumped peristaltically using Watson-Marlow flow inducers (the model reflecting required pump rates). Glass and flexible pvc tubing were used, short sections of silicone rubber tubing only being used in the pump head, due to eventual stretching and splitting of pvc tubing at this point. No silicone rubber was used during growth of strain ALV (4.4.3). Marsh (1985) observed toxicity to strain TH1 in the presence of silcone rubber tubing and in comparative tests found the pvc tubing to be a non-toxic flexible alternative. Hence, before using strain ALV in a continuous flow system (4.4.3) it's response to silicone rubber during growth was tested. In growth curves for 100 ml cultures with 50 mM ferrous iron, lengths of silicone rubber tubing in the medium prevented oxidation the limited oxidation being equivalent to that of a sterile control (fig 2.3). Flexible pvc tubing in the medium allowed complete oxidation but with a more rapid decline in oxidation rate than with a tubing free control. This was consistent both with addition of pvc tubing and the addition of tubing followed by autoclaving. (This latter step was to remove any volatile compounds leached from the tubing). The presence of pvc tubing had no effect on T.ferrooxidans. Flexible pvc, however, did not consistently survive autoclaving if connected to other apparatus (splitting at the join) and so was sterilized separately and re-connected in aseptic conditions. Flow rate into the vessels was measured over time by collecting inflow in measuring cylinders, for unsterile conditions (eg 4.4.1), measuring the overflow collected daily (4.4.4) or via a pipette placed vertically in the feed line.This, when filled under pressure became a calibrated reservoir pumped out when the main reservoir was temporarily clamped

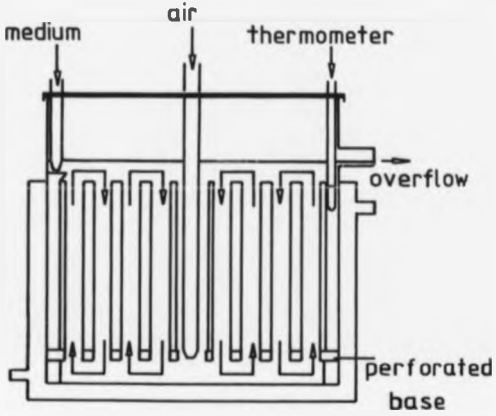


Fig 2.2
Cross section diagram of a lab-scale model of the Bacfox process (experimental detail 4.4.2). The glass vessel was fitted with a glass water jacket. The side arm in the wall of the vessel, above the level of the water jacket was used as a weir type overflow. The neck of the vessel was covered by a plastic lid, cut to allow access of fresh medium, dripped into the vessel; air, bubbled in at the vessel base; and a thermometer. (This reduced evaporation during non-sterile operation). Internally the vessel was fitted with glass tubes through which fresh medium and air were circulated (indicated by arrows). Each tube was closely fitted to its neighbours reducing the space between them (each marked solid wall is a composite of two separate glass walls). The tubes were individually matched to holes drilled in a perspex plate, suspended above the base allowing both for homogeneous mixing and circulation of the liquid medium, air and suspended bacteria.

off. Inaccuracies were found in this method at high flow rate and with a blow-out overflow (4.4.4). The waste media from all vessels was collected in waste pots, sealed and filtered to prevent contaminating aerosols forming.

2.2.4 Measurement of growth

Cell growth was measured both by the extent of ferrous iron oxidation and the degree of mineral dissolution. Residual concentrations of soluble ferrous iron were measured by titration. Accurately measured 1 ml samples were placed in 2 ml of 5% v/v sulphuric acid to halt oxidation and titrated at room temperature against 0.005 N ceric sulphate (ie 5 mM) prepared in 5% v/v sulphuric acid. Each titration was corrected for ferrous iron present in the single drop of 1,10 phenanthroline-ferrous sulphate complex used as the end point indicator. The indicator usually consumed 0.3 ml ceric sulphate. Correction of values allowed conversion to specific ferrous iron concentration, each ml consumed representing 5 mM ferrous iron present in the sample. With smaller concentrations of ferrous iron larger sample volumes were taken. During batch growth with varying ferrous iron substrate concentrations (5 mM to 50 mM - 4.3) the sample size was 2.5 ml. A total volume of 5.5 to 6 ml was removed and on cooling to room temperature (where necessary) accurate samples of 2.5 ml were titrated in duplicate. Alternatively, with 25 mM ferrous iron during growth with ferric iron, where overall growth would be slower (hence allowing for more samples) the total sample volume was reduced by measuring 1 ml samples in duplicate against diluted ceric sulphate, 2.5 mM. Samples from mineral grown cultures were prespun in a micro-angle centrifuge to remove excess mineral prior to titration. Final sample volumes were measured at room temperature.

Spun samples were also used for measurement of total soluble metal concentrations, 0.2 ml being diluted in 9.8 ml of 1% v/v hydrochloric acid and stored in capped scintillation vials. Vials were kept at 4°C for prolonged storage, the samples being measured in an acetylene flame atomic absorption spectrophotometer. Each series of

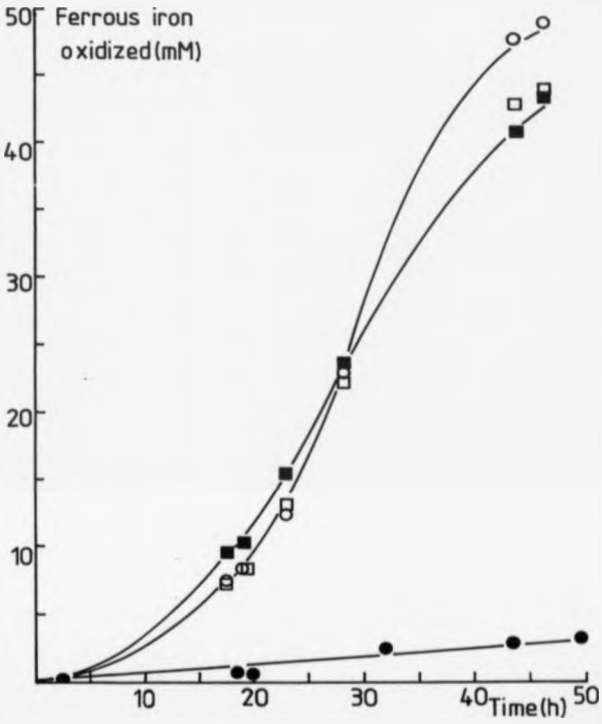


Fig 2.3
Iron oxidation during batch growth of strain ALV. Flasks (250ml) containing enriched salts (100ml), pH1.7 were shaken (120rpm) at 45°C, gassed with 1% v/v CO_2 in air. Flasks contained 50mM ferrous iron, only(○) and 15 lengths(1.5cm) of tubing: pvc(□), pvc autoclaved with the medium(■), and silicone rubber(●, identical to sterile control, not shown). Inocula were 5% v/v.

measurements included a standard calibration dilution series. Stock standard solutions were prepared using analytical balances and chemicals and acid clean volumetric glassware with 1% v/v hydrochloric acid as the diluent. Readings for the standard series of 10, 20, 50 and 100 μM metal were repeated after every 10 samples and with a blank correction to allow for drift in the values. Standards were used to convert machine readings to concentration values. Samples were diluted if they exceeded the range of the standards (this was allowed for in the final correction). With mineral grown cultures in shake flasks, each flask was weighed prior to sampling.The difference between consecutive weights, allowing for total sample volume removed was used an an indicator of medium loss by evaporation, 1 ml being equivalent to 1 g weight loss. Percentage loss was used to correct concentration readings for soluble metals.

Protein was used to express organism concentration and was used less regularly to measure growth. Protein concentration was measured on washed cell suspensions, prepared for oxidative studies (2.3) or specifically for protein measurement. With iron grown cultures the sample size for protein estimation was up to 100 ml volume depending on the degree of oxidation (during continuous culture) and therefore expected cell yield. Samples were centrifuged, the pellets resuspended in distilled water and centrifuged to wash the cells (see 2.3). This removed soluble iron which interfered with the colourimetric assay. Samples from mineral grown cultures were slowly spun in micro-angle centrifuge tubes to deposit residual mineral particles. Usually repeated three times this was observed to incur associated cell loss and underestimates of protein concentration. The final pellets from any given sample were bulked and resuspended to a total 0.5 ml volume in water, and made up to 1 ml in 0.1 M sodium hydroxide. These whole cell suspensions were disrupted by boiling for 5 minutes in sealed test tubes. A standard protein series was run with each determination using an aqueous bovine serum albumin suspension diluted to 1 ml at 10, 20, 50, 100, 150 and 200 μg per ml final

concentration. To each tube was added 5 ml solution A, which was mixed and stood for exactly 10 minutes. Then 0.5 ml solution B was added, whirlimixed, stood for exactly 30 minutes and measured spectrophotometrically against a reaction blank at 700 nm. Solution A was 1 ml each of 2% w/v sodium potassium tartrate and 0.5% w/v hydrated copper sulphate (both aqueous) in 98 ml 2% w/v sodium carbonate (prepared in 0.1 M sodium hydroxide). Solution B was a mixture of Folin-Ciocalteu phenol reagent and water in a 1 to 2 ratio. Solutions A and B were prepared fresh from stock solutions for each determination.(This assay was an adaptation used by Marsh,1985).

2.2.5 Measurement of conditions - pH

Measurement of pH was by a glass electrode used exclusively at acid pH (below pH 3). The instrument was routinely calibrated between pH 1.3 and pH 2 at 97% to 99% sensitivity (ie predicted accuracy which decreased with age of the electrode). Calibration used solutions of constant ionic strength and pre-determined pH value. A commercial glycine buffer of pH 2.02 was also used. Meter readings were compensated for changes in ambient temperature to which all culture samples were pre-cooled prior to reading. Table 2.3 presents the chemical proportions for the pH standard solutions.

TABLE 2.3

Composition of solutions of known pH and constant ionic strength. Prepared in 100 ml of water proportions are of x ml 0.2 M hydrochloric acid and of y ml 0.2 M potassium chloride.

pH	x	y	pH	x	y
1.2	40.7	9.3	1.7	12.8	37.2
1.3	32.3	17.7	1.8	10.2	39.8
1.4	25.7	24.3	1.9	8.1	41.9
1.5	20.1	29.9	2.0	6.5	43.5
1.6	16.0	34.0			

2.3 CELL HARVESTING AND PREPARATION

All cells were centrifuged to prepare suspensions by the same method irrespective of their final use in the oxygen electrode (Chapter 4) or in respiratory chain studies including preparation of cell free extract (Chapter 5). Differences in equipment used only reflected total medium volume to be harvested.

2.3.1 Whole cell suspensions

Bulk biomass was grown in batch culture with 50 mM ferrous iron. For each oxygen electrode experiment (per organism) growth was in 3 or 4, 2 l shake flasks with 1.5 l medium in each (2.2.2). Volume was increased to 3 or 4, 20 l glass carboys to provide cells for the respiratory chain studies (2.2.2, Chapter 5). Iron grown cultures were harvested in late exponential growth phase - greater than 80% ferrous iron oxidized. With flasks, 6 x 400 ml capped nalgene pots were spun in Beckman centrifuges at 15,000 rpm for 15 min. Mineral grown Sulfolobus BC65 cultures were pre-settled and the supernatant siphoned off for centrifugation. The tens of litres of media from the carboys were fed by siphon into a Westphalia continuous action centrifuge. After the initial centrifugation the cell pellets or cell paste were resuspended in water acidified with sulphuric acid to pH 1.7 and where appropriate bulked together. These suspensions were centrifuged prior to two further resuspensions and washings by centrifugation. (A reduced scale procedure was used on protein samples, 2.2.4). The same method was also used to wash cell inocula to remove substrate carry-over prior to measuring growth curves (3.2). The final washed pellets for use in the oxygen electrode were resuspended to a final optical density of 0.7 using a Corning colourimeter at a wavelength of 540 nm for all the organisms. With the bulk biomass required in the respiratory chain studies the final pellets were frozen wet until sufficient quantity was bulked together. After thawing these pellets were resuspended in beta-alanine sulphate buffer, 50 mM at pH 3.5 to an approximate

dilution of 1 in 4 ie 25% w/v.

2.3.2 Cell free extract

Freshly harvested or freeze-thawed washed cell pellets were prepared at 10% w/v concentrations in pH 2 water prior to cell disruption for extract preparations. Each suspension was kept on ice during passage through a French pressure cell press, several times at 18,000 psi pressure. T.ferrooxidans appeared sufficiently disrupted after three passages. However, L.ferrooxidans appeared to stick in the pressure cell and as a result frequently passed through the cell at greatly reduced pressure and hence was pressed during five consecutive passages. Gross cell debris and remaining whole cells were removed from the crude extracts by centrifugation at 10,000 rpm for 10 minutes. This was repeated on the supernatant. Both organism supernatants were then subjected to an ultracentrifugation spin using a Beckman preparin set to spin at 140,000 g for 1 hour. During use the final extracts were kept on ice or at 4°C.

2.4 MEASUREMENT OF IRON OXIDATION BY CELL SUSPENSIONS

Cell suspensions were prepared as in 2.3.1 and their ability to oxidize ferrous iron measured using an oxygen electrode and by Warburg manometry. The results and the analysis of data (as chart recorder traces) were influenced by biological responses and hence are discussed as part of the results (4.2.1). The equipment and experimental design were standardized.

2.4.1 Oxygen electrode - operation and calibration

The oxygen electrode was a Clark type instrument composed of silver and platinum electrodes mounted in perspex and surmounted by a water-jacketed perspex reaction chamber. The reaction chamber was sealed with a central perspex stopper incorporating an injection point in the centre. Before each new experiment the metal electrodes were polished with silver polish, flooded with fresh saturated potassium chloride solution as electrolyte and covered

with a fresh square of teflon membrane. This whole assembly was mounted on a magnetic stirrer, the small magnetic follower running on the membrane within the reaction chamber. The polarisation voltage across the electrodes was 0.6 volts and the reaction temperature was maintained by a circulating water heater. Oxidation within the reaction chamber caused changes in electrical potential of the system, followed as continuous traces on a single channel paper chart recorder. Calibration of the chart recorder expressed change in electrical potential as removal of oxygen from solution as a percentage. Normal calibration of an oxygen electrode was via the oxidation reaction involving a catalase enzyme and NADH under darkened conditions.* However for the conditions of the acidophiles NADH was not stable at pH 1.7 and particularly at 65°C (for Sulfolobus BC65) the catalase was inactivated by raised temperature. (Progressive use under these conditions caused deposition on and poisoning of the electrodes - hence the polishing). The electrode was calibrated by a more empirical method which allowed for standardization of the procedure. All reactions were of a final 3 ml volume at pH 1.7. Using acidified water blanks at the relevant reaction temperature (30°C, 50°C or 65°C) a stirred 3 ml volume was taken as having 100% of the theoretical oxygen dissolution value. Before each experiment this was registered at 95 chart divisions on the recorder. This oxygen content was stripped by the addition of grains of sodium dithionite, the response of the chart recorder being correspondingly set at 0 chart divisions. Therefore, for each experiment, the chart recorder was calibrated at 0% and 100% responses for air saturated blanks, the specific oxygen content of which decreased with increasing temperature. Each chart division hence represented a specific oxygen concentration, uptake of which was measured by calibrated chart speed. Conversion of rate to specific concentration values had three components, (1) oxidation rate changed with cell concentration used, (2) oxidation rate was measured initially as x chart divisions per minute, (3) oxygen content of reaction mix and therefore of each chart

* Beechey and Gibbons(1972)

division was temperature dependant. Hence, the units to express specific rate were micromoles molecular oxygen consumed per minute of reaction per mg bacterial protein present (ie μ mol $O_2 min^{-1} mg^{-1}$). (The estimations of specific oxygen content used in calculations at each reaction temperature are outlined in appendix 2).

All the components of a reaction except the cell suspension(4.2.1) were placed in the oxygen electrode and equilibrated over a pre-set time until both temperature and the 100% oxygen response on the chart recorder were reached. A ferrous iron solution of 278 g per litre ferrous sulphate was prepared fresh, unsterile at pH 1.7 for each experiment. From this a dilution series was prepared allowing 300 microlitres of each dilution to give in 3 ml, a ferrous iron concentration of 0.66 mM, 1 mM, 2 mM, 5 mM, 10 mM, 20 mM and 50 mM. Ferric iron solutions (generally 50 mM) were prepared with and without pH control (as in 2.2.1). The final concentration used was 10 mM (unless stated). Each individual reaction was initiated by the addition of cells whilst the stopper was positioned to remove all air spaces from above the total liquid volume.

2.4.2 Oxygen electrode - graphical presentation and analysis of data

In each experiment two rate determinations were made at each substrate concentration, plus a third determination in the presence of an inhibitor. Extra determinations for one substrate concentration (50 mM) were used in calibration curves of percentage activity loss over time. Rates were subsequently corrected where necessary. Each rate was measured for a linear stretch of the chart recorder plot (response in the oxygen electrode was organism specific, 4.2.1) as chart divs per min and was converted to a specific rate (as in 2.4.1) designated V. Substrate concentrations in mM were designated S. Duplicate determinations of V were averaged. These values, directly and as mathematical derivatives were used to construct three graphical plots for each experiment based on linear forms of the Michaelis - Menten equation.

(Examples of each plot are given for T.ferrooxidans in fig 4.6). Firstly, the double reciprocal plot was reciprocal values of V plotted on the ordinate axis against reciprocal values of S on the abscissa. The straight line relationship cut the ordinate axis at the reciprocal value of V_{max} and the abscissa at the negative reciprocal of K_m (fig 4.6). Alternatively the value of the slope was V_{max} divided by the K_m. During competitive inhibition the intersection on the ordinate did not change but that on the abscissa did, becoming the negative reciprocal of K_p. This could be used to find the inhibitor constant K_i from equation 2.1, where i is the concentration of inhibitor and as with all the other constants is in mM.

$$K_i = \frac{i}{(K_p/K_m)-1} \qquad 2.1$$

Alternatively the K_i could be measured by the alteration in K_m during inhibition as in equation 2.2, where K_m' was measured in the absence of inhibition. This relationship

$$K_m \text{ (during inhibition)} = K_m' (1 + i/K_i) \qquad 2.2$$

also held for the slopes of the two lines formed for double reciprocal plots for iron oxidation in the presence and absence of ferric iron inhibitor. It was these values of slope(b) replacing K_m in equation 2.2 that were used to calculate K_i. Secondly, values of S/V on the ordinate axis were plotted against S on the abscissa, the slope of the linear relationship being the reciprocal of V_{max} and the intersection on the abscissa being the negative value of K_m. Thirdly, V on the ordinate was plotted against V/S on the abscissa, the intersection of the linear relationship on the ordinate axis being V_{max} and the value of the slope being the negative K_m. The line for all the graphical plots was drawn on the basis of a calculated linear regression equation of the form $y = a + b\,x$ derived for each individual plot where x and y were the data

points on the abscissa and ordinate axes respectively. These values of b were used as above in calculating K_i from double reciprocal plots. Calculations were made on a programmed calculator incorporating a statistical linear regression function. Once derived the mean value was taken of all the K_m or K_i values for one organism and presented with the standard deviation for n number of data points.

2.4.3 Warburg manometry

Respiration of the moderate thermophiles was also measured using cell suspensions in a heated rotating Warburg bath fitted with dye-filled manometers. The suspensions were placed in the side arms of Warburg flasks which were fitted with seals on to the manometers and agitated whilst equilibrating to the temperature in the bath. As oxygen was consumed changes in pressure affected the heights of the dye in the manometer. An empty flask was used as a control registering change due to atmospheric pressure changes (thermobarometer). Final flask volumes were 2.5 ml. After equilibration to 45°C the cells were tipped from the side arms to start the reaction and pressure changes were measured over time as total change in mm of the dye height within the manometer. These figures were corrected for the atmospheric control and corrected to specific values of oxygen uptake (as microlitres per unit time). Protein concentration and cell volumes and concentrations of substrate used are given in the figure legends (figs 4.3,4.4).

2.5 SPECTROPHOTOMETRY OF THE RESPIRATORY CHAIN

2.5.1 Whole cells

Freeze thawed cell suspensions in buffer were prepared as in 2.3.1. Volumes of 1.25 ml were scanned in narrow black-sided glass cuvettes of 1 cm path length at wavelengths between 400 nm and 700 nm in a dual beam spectrophotometer. Cell-suspensions were kept on ice and when required each cuvette was oxidised with 10

microlitres of a 1 mM hydrogen peroxide solution or reduced with a few grains of sodium dithionite. Measured at room temperature a baseline was measured of two identical oxidised samples which was then used as the correction for a reduced minus oxidised difference spectrum, on adding dithionite to one of the samples and repeating the scan. This procedure was repeated at low temperature by freezing the samples at liquid nitrogen temperature and holding them at this temperature whilst scanning between 500 nm and 700 nm. (Results are discussed in Chapter 5). At room temperature baselines were also measured for reduced samples after which one sample was bubbled for exactly 2 min with pure carbon monoxide (via a pasteur pipette). Re-run, the scan hence indicated the effect of any cell binding of carbon monoxide.

2.5.2 Cell free extracts

Cell free extracts (2.3.2) were measured spectrophotometrically either as absolute spectra against water blanks in glass cuvettes of 1 cm pathlength at 30°C, or as difference spectra at room temperature in the same conditions as 2.5.1. Pyridine derivatives of L.ferrooxidans extract were prepared by adding the cell free extract to a solution with final concentrations of 3 M pyridine anhydride and 0.2 M sodium hydroxide.

2.6 CHEMICALS AND EQUIPMENT

2.6.1 Chemicals

The chemicals used in this study were purchased from the following suppliers:

(1) BDH Chemicals Ltd, Poole

Ammonium sulphate	Sodium hydroxide
Potassium chloride	Cupric sulphate (hydrated)
Calcium nitrate	Sodium thiosulphate (hydrated)

1,10 - Phenanthroline-ferrous sulphate complex 0.025M

(2) Fisons plc, Loughborough

Magnesium sulphate — Potassium sodium tartrate

Glucose — Sulphuric acid (S G 1.84)

Ferrous sulphate (hydrated, GPR and AnalaR)

(3) May and Baker Ltd, Dagenham

Ceric sulphate — Hydrochloric acid

(4) Hopkins and Williams, Chadwell Heath, Essex

Ferric sulphate (hydrated - GPR)

(5) Fluka A G, Switzerland

Potassium tetrathionate

(6) Sigma Chemicals

Bovine serum albumin, crystalline anhydrous

(7) LABM Ltd, Adelaide House, London Bridge

Powdered yeast extract

All the above chemicals were anhydrous or AnalaR grade unless stated. All gas cylinders were supplied by BOC.

2.6.2 Equipment

The following equipment was used in the experimental work throughout this study. Atomic absorption spectrophotometry was measured on a Rank Hilger H 1550, a Varian AA - 1275 series fitted with an ancillary printer and model S5 programmable sample changer and a Shimadzu model spectrophotometer, pre-programmable and with built-in printer. Routine optical densities and protein determinations were measured by a Corning colorimeter and a Pye Unicam SP 600 spectrophotometer. Dual beam spectrophotometers used were Pye Unicam SP 1800 and SP 6-200 models and a Perkin-Elmer Lambda 5. Measurements at liquid nitrogen temperature were in a specifically built split beam research instrument at the Dept Biochemistry and Microbiology, St Andrews University. The oxygen electrode was supplied by Rank Bros, Bottisham, Cambridge and fitted to a Kipp and Zonen BD 8 recorder. The circulating water pumps were from Conair Churchill Ltd, Uxbridge, Middlesex. All centrifuges were Beckman models or a B and T micro angle centrifuge. All the peristaltic

pumps were from Watson Marlow Ltd, Falmouth, Cornwall, MHRE models and an HR flow inducer. The magnetic stirrer/hot plates were models from Gallenkamp or Heidolph. Overhead electric motors were Heidolph RZR1 or IKA-WERK from Janke and Kunkel and Co GmbH. During the grading of ground mineral, separation was in a Warmen Cyclosizer (Simon Warmen Ltd, Lancs) the size being measured in a Malvern Laser Droplet particle sizer 2600 D (Malvern Instruments Ltd, Spring Lane, Malvern, Lancs). The glass fermenter vessels were made by Cutforth glass blowers, Birmingham. Samples for total carbon analysis were measured in a Beckman total carbon analyzer.

3 PHYSIOLOGY OF FERROUS IRON OXIDATION

3.1 INTRODUCTION

Physiological studies of ferrous iron oxidation have principally involved the organisms binomially designated T.ferrooxidans and L.ferrooxidans. This investigation concentrated on a comparison of the physiologies of the relatively recently isolated and as yet unnamed moderately thermophilic strains. Specifically, strains TH1, BC1, TH3, ALV and LM2 were studied (isolation and designation - 2.1). Less detailed comparison was also made with Sulfolobus BC65. In particular, identification was sought of a thermophilic organism capable of ferrous iron oxidation in the same cultural conditions as T.ferrooxidans. Comparison would then be made of such an organism and T.ferrooxidans in continuous ferrous iron culture. Thermophiles largely required supplements to their media, of reduced sulphur, not required by T.ferrooxidans (1.3.3,1.8.2). The investigation reflected the need for these growth requirements and attempted to more closely define obligation for and response to such supplements by each organism. The types of supplements used were also designed to exhibit different types of nutritional growth patterns present in the various organisms. Utilizing different types and concentrations of supplement, the main characteristics measured for each strain were the rate and degree of ferrous iron oxidation. Where possible, stock cultures for experimental use were maintained under chemolithotrophic conditions, with the minimum of additions to the basic ferrous iron, enriched salts medium (2.2.1). Restricting non essential additions, although possibly reducing the potential maximum growth rate, reduced the risk of substrate carry over with inocula into a serial sub-culture in a defined medium. Composition of stock culture media had, though, to be altered throughout the study as a result of

experimental data, to maintain these minimal culture conditions. Results therefore, were based on more than one experiment. Where appropriate, experimental conditions were directly repeated via sub-culture to illustrate the consistency of organism response to nutritional supplements. The concentration of supplements used (table 2.2) was not uniform (experimental details are summarized in each figure legend). Grown in shake flasks, batch cultures were supplied with carbon dioxide enriched air (1% v/v) except on the addition of yeast extract at mgl^{-1} . Although known to be in excess, the physiological studies did not include the measurement of the required minimum concentration of carbon dioxide in air to support organism growth in these defined conditions. The basic unsupplemented medium was 100 ml enriched salts at pH 1.7 containing 50 mM ferrous iron (materials and methods, 2.2.1).

3.2 EFFECT OF GROWTH SUPPLEMENTS - MODERATE THERMOPHILES

3.2.1 Strain TH3

The re-isolated TH3 strain (2.1) did not oxidise ferrous iron in chemolithotrophic conditions. Irrespective of reduced sulphur supplementation, chemolithotrophic growth and iron oxidation in batch culture were minimal or largely incomplete. This was a consistent phenomenon despite the use of carbon dioxide enriched air. Growth curves, of the concentration of ferrous iron oxidised against time in various supplemented media, are illustrated in fig 3.1. Without any supplements to the basic salts and ferrous iron medium, the level of ferrous iron oxidation equalled that of the sterile control. Oxidation did occur with reduced sulphur present (1mM tetrathionate) but this was partial and incomplete relative to the concentration of ferrous iron available. This partial level of oxidation was absent with 1mM glucose. Combined with the carbon dioxide enrichment, this glucose concentration would have been expected as capable of supporting mixotrophy. Therefore, by

implication, strain TH3 was not capable of mixotrophic utilisation of ferrous iron substrate. Rapid growth and complete iron oxidation were supported by yeast extract at 100 mgl^{-1}(fig 3.1). This chemolithoheterotrophic growth did not require gassing of the cultures with carbon dioxide enriched air, but the extent of growth was dependent on the concentration of yeast extract. In the presence of 1 mgl^{-1} yeast extract, the degree of ferrous iron oxidation was comparable to that with 1mM tetrathionate, albeit with a slightly reduced lag phase (fig 3.1). Such limited oxidation was significantly extended when both supplements were simultaneously present. Not only the rate but the extent of oxidation, with both 1mM tetrathionate and 1mgl^{-1}yeast extract together were increased, and the total ferrous iron oxidized appeared greater than the sum of the concentrations oxidized with the individual supplements present separately. Adding 1mM glucose suppressed this apparent complementaton of yeast extract and tetrathionate. Under these conditions oxidation initially comparable to that measured with either yeast extract or tetrathionate , failed (fig 3.1). In some experiments the presence of 1mgl^{-1}yeast extract could support oxidation of up to 70% of the available ferrous iron (fig 3.2). In such circumstances the complementary addition of reduced sulphur, 200 mgl^{-1} sodium thiosulphate (1.265 mM), had little effect on the overall percentage of iron oxidized (fig 3.2) The presence of thiosulphate alone had a limited effect on iron oxidation, being similar in degree to ferric iron yields with 1mM tetrathionate (figs 3.2, 3.1). Such limited oxidation was above sterile control levels and was again suppressed in the presence of glucose. Therefore, rather than just not supporting iron oxidation, glucose appeared to be an active inhibitor of ferrous iron oxidation in strain TH3 (fig 3.2). Alternatively, reduced sulphur irrespective of the form in which it was supplied could support limited oxidation even to the point of complementing a growth limiting yeast extract concentration, although this latter point was not a consistent phenomenon.

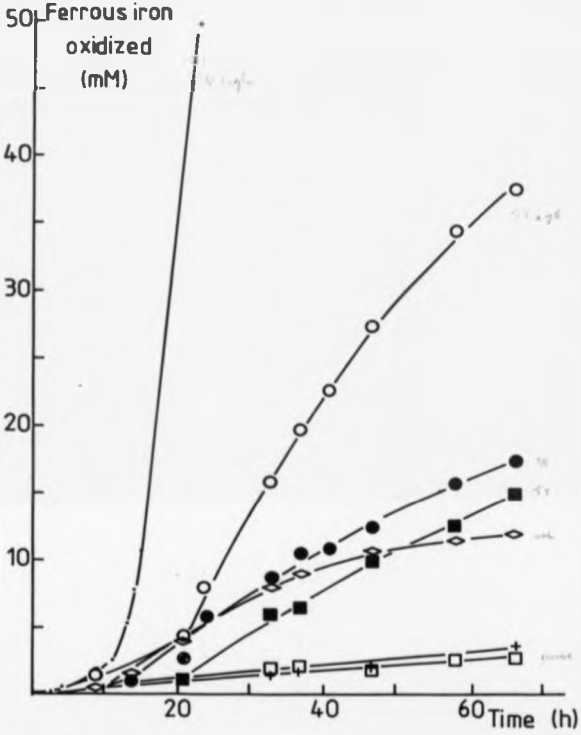


Fig 3.1
Batch growth curves for strain TH3, in 100ml ES at 50°C, pH1.7 in shake flasks(250ml vol) at 120rpm with 50mM ferrous iron and: glucose,1mM(□), YE,1mgl^{-1}(●), 100mgl^{-1}(·)*, tetrathionate,1mM(■), tetrathionate,1mM and YE,1mgl^{-1}(○), tetrathionate, 1 mM, YE, 1mgl^{-1} and glucose, 1mM (◇). Growth without supplement (+) identical to abiotic control (not shown). Inocula, 5%v/v were from a 2mgl^{-1} YE supplemented culture. Flasks were trickled with 1% v/v CO_2 in air, except * - normal diffusion.

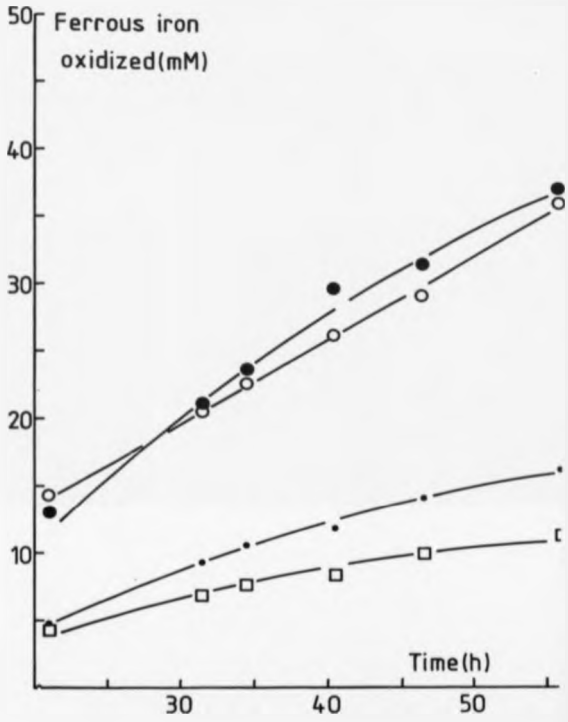


Fig 3.2
Batch growth curves for strain TH3, in 100ml ES at 30°C, pH 1.7 in shake flasks(250ml vol) at 120rpm with 50mM ferrous iron and: $1mgl^{-1}$ YE(○), $200mgl^{-1}$ thiosulphate(●). $1mgl^{-1}$ YE and $200mgl^{-1}$ thiosulphate(◐), $200mgl^{-1}$ thiosulphate and 1mM glucose(□). Inocula, 5%v/v were from a $1mgl^{-1}$ YE supplemented culture. Flasks were trickled with 1%v/v CO_2 in air.

3.2.2 Strains TH1 and BC1

Based on comparative protein gel patterns for whole cells (Marsh, 1985), strains TH1 and BC1 were putatively identified as different strains of the same species. Both strains were used individually in growth curve experiments to provide physiological evidence for their synonymous nature . (Experimental results were published in part, Norris and Barr, 1985. To avoid duplication in the presentation of similar data, the particular isolate used is noted in the appropriate figure legend illustrating each experimental result, but where in the text the experiment is referred to for the alternative isolate, the duplicate figure for that isolate is presented in appendix 3).

Strain BC1 did not consistently oxidize ferrous iron in the absence of reduced sulphur. However, ferrous iron oxidation did occur in the first sub-culture from a tetrathionate supplemented culture into fresh medium free of reduced sulphur. The extent of oxidation was limited to approximately 60% of the available ferrous iron (fig 3.3; a similar figure is presented for strain TH1 in appendix 3). Subsequent inoculations from this growth limited culture consistently failed to grow in the absence of reduced sulphur. In the presence of 1mM tetrathionate iron oxidation was rapid, complete and sustainable through serial sub-culture (fig 3.3). The initial limited oxidation in the absence of added reduced sulphur in the first sub-culture appeared then to be due to excess reduced sulphur (tetrathionate) carried over with the cell inoculum. As neither strain BC1 nor strain TH1 could be continuously sub-cultured on ferrous iron without reduced sulphur, washed cell inocula were used in investigating this phenomenon. Using a micro-angle centrifuge, cell inocula from grown cultures were spun and washed in acidified, distilled water to remove excess substrate. Inocula were re-suspended to the original volume in fresh sterile salts solution without any ferrous iron or reduced sulphur (method, 2.3.1). Ferrous iron oxidation was measured using washed cell inocula for strain TH1 in the presence of concentrations of 0, 1, 10, 100 and 500 μM

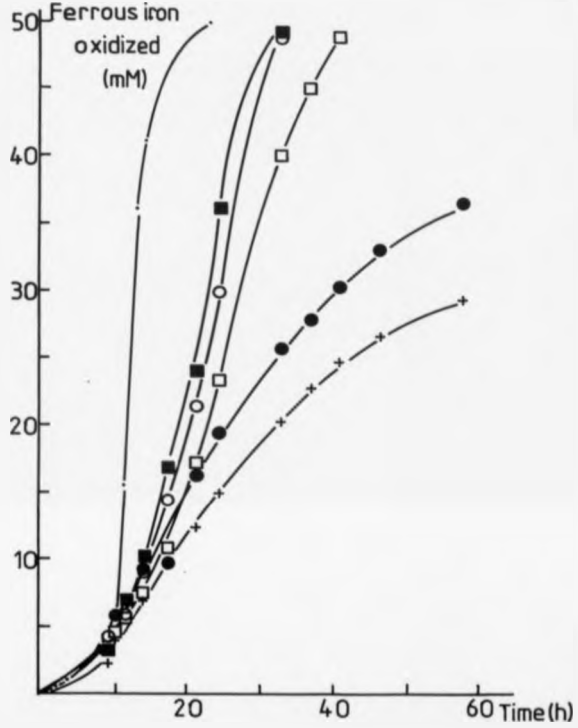


Fig 3.3
Batch growth curves for strain BC1, in 100ml ES at 30°C, pH 1.7 in shake flasks(250ml vol) at 120rpm with 50mM ferrous iron, only(+) and plus: $1mgl^{-1}$ YE(●), $100mgl^{-1}$ YE(·) *, 1mM glucose(□), 1mM tetrathionate(■), 1mM tetrathionate and $1mgl^{-1}$ YE(○). Inocula, 5%v/v were from 1mM tetrathionate supplemented culture. Flasks trickled with 1% v/v CO_2 in air except * - no air. (Complementary plots for strain TH1 presented in fig A3.1).

tetrathionate (fig 3.4.). In the absence of tetrathionate, with strain TH1, no oxidation occurred beyond that observed in the abiotic controls, but both the rate and extent of oxidation subsequently increased with increasing initial tetrathionate concentration, complete oxidation occurring in the presence of 100 µM tetrathionate (fig 3.4). The concentration increase from 100 µM to 500 µM tetrathionate allowed an increase in the maximum attained oxidation rate which was also sustained for longer. These levels of tetrathionate indicated that the original use of 1 mM tetrathionate for growth was in excess to that required to ensure complete oxidation of 50 mM ferrous iron. Further, the degree of oxidation with 10 µM tetrathionate and a washed cell inoculum, though incomplete, was comparable to the degree of oxidation in the first sub-culture without reduced sulphur (figs 3.3, 3.4). That is, the concentration of reduced sulphur required to be carried over to ensure this level of oxidation in reduced sulphur free medium was some 1% of the initial supplement, itself known to be present at a concentration at least ten times greater than that needed for complete oxidation. Similar ferric iron yields at the corresponding tetrathionate concentrations were also seen with strain BC1 (appendix 3). Neither the mechanism of sulphur incorporation nor the final form of sulphur that was assimilated were investigated. However, the requirement for reduced sulphur though obligate (in the absence of yeast extract) was not compound specific to tetrathionate. Both elemental sulphur and thiosulphate could be used to support complete oxidation (by strain BC1) in the absence of tetrathionate (fig 3.5). The growth curve with thiosulphate had a greater lag phase than with tetrathionate. This compound was also rate limiting relative to it's concentration and in the experiment of fig 3.5 the tetrathionate concentration represented a greater concentration of sulphur than present in the thiosulphate. With the flowers of sulphur, though the sulphur was in excess, the solid nature of it's form resulted in a much slower growth curve overall.

Glucose (1 mM) sustained complete oxidation of ferrous

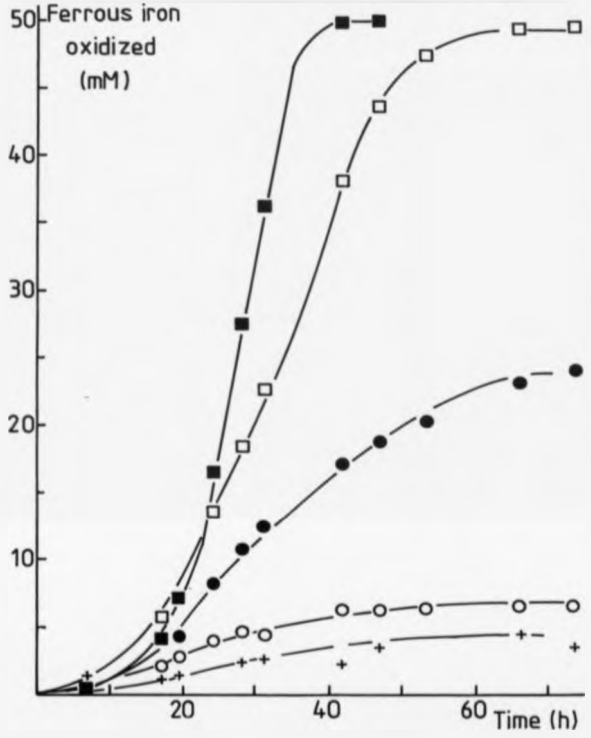


Fig 3.4
Batch growth curves for strain TH1 with washed cell inocula (3.5% v/v). Shake flasks(250ml vol) at 30°C, 120rpm contained 100 ml ES, pH 1.7 with 50 mM ferrous iron and tetrathionate concentrations of 0uM(+, identical to abiotic control, not shown) 1uM(○), 10uM(●), 100uM(□) and 500uM (■). Inocula were from a 0.1mM tetrathionate supplemented culture. Flasks were trickled with 1%v/v CO_2 in air. (Complementary curves for BC1 in fig A3.2).

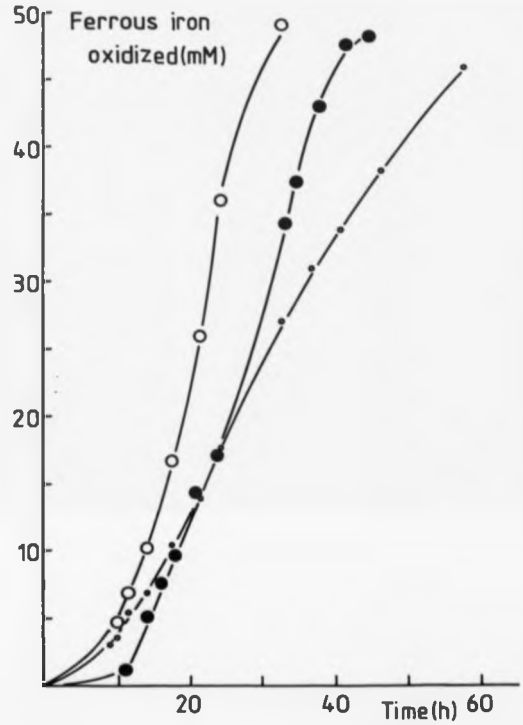


Fig 3.5
Ferrous iron oxidation by strain BC1 in 100ml batch culture at 30°C with different reduced sulphur supplements. Inocula, 5%v/v from tetrathionate supplemented culture were placed in ES medium, pH1.7 with 30mM ferrous iron and tetrathionate, 1mM(O), thiosulphate, 1.265mM(●) and 100mgl^{-1} elemental sulphur flowers(•). Flasks(250ml vol) shaken at 120rpm were trickled with 1% v/v CO_2 in air.

iron by strain BC1 (fig 3.3) and in the absence of added reduced sulphur. Compared with 1 mM tetrathionate the maximum rate of ferrous iron oxidation declined slightly in the latter stages with 1 mM glucose. Neither the rate nor the extent of glucose supported oxidation were sustainable through serial sub-culture. Having shown that all residual reduced sulphur was expended in the first sub-culture, this failure of glucose to support subsequent iron oxidation suggested that glucose stimulated oxidation was dependant on the presence of reduced sulphur. That glucose was stimulating growth was evident in that the carry-over of reduced sulphur could not itself sustain complete oxidation, as seen in fig 3.6 and that the degree of stimulation both in the increased degree and rate of iron oxidation was concentration dependant for glucose (strain BC1 , fig 3.6). This was measured in the first sub-culture without added reduced sulphur. The minimum level of reduced sulphur required for complete oxidation with glucose was not investigated. When glucose and a concentration of reduced sulphur, sufficient to support full oxidation individually (in the first non supplemented sub-culture), were present together, the combined effect was to slightly reduce the lag phase and delay the final decline in maximum oxidation rate relative to the respective individual growth curves.

The maximum iron oxidation rates with both strains BC1 and TH1 were in the presence of 100 mgl^{-1} yeast extract (figs A3.1; 3.3). The corresponding growth curve rapidly attained the maximum oxidation rate which only declined as the oxidation of the available ferrous iron substrate neared completion. This level of oxidation was due to chemolithoheterotrophic growth by these strains. (Heterotrophic growth of strain TH1 on yeast extract alone would yield significantly less cells, as measured by optical density, compared to this iron-yeast extract grown culture, Marsh, 1985). Ferrous iron oxidation in the presence of $1mgl^{-1}$ yeast extract was though both slow and incomplete. Comparable to the non supplemented oxidation rate in the first sub-culture the absence of any complementary effect of $1mgl^{-1}$ yeast extract on the growth

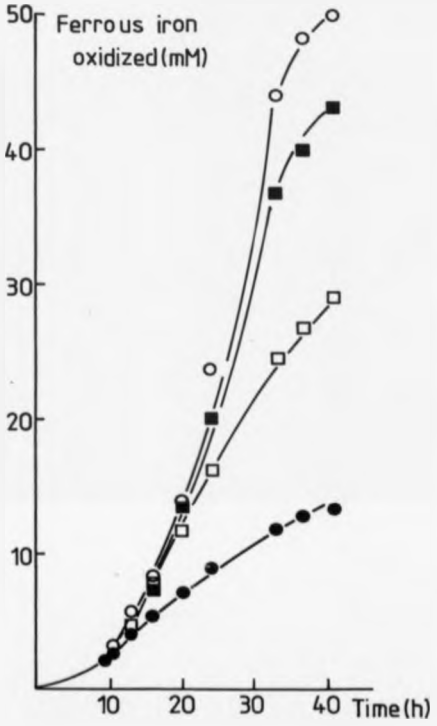


Fig 3.6
Effect of glucose concentration on growth curves of strain BC1. Shake flasks(250ml vol shaken at 120rpm) were inoculated 5%v/v from a tetrathionate supplemented culture. Reduced-sulphur free medium (100ml ES. pH 1.7 with 50mM ferrous iron) contained 0mM(●), 0.01mM(□),0.1mM(■) and 1 mM(○) glucose. Flasks were aerated with a trickle of 1% v/v CO_2 in air.

curve of strain BC1 supplemented with 1 mM tetrathionate (fig 3.3) suggested that this concentration was insufficient to significantly stimulate iron oxidation. Unlike glucose, no reduced sulphur was required for sustained oxidation through sub-culture with 100 mgl^{-1} yeast extract in the ferrous iron-mineral salts medium.

3.2.3 Strain LM2

Strain LM2 was routinely grown with 1 mM tetrathionate in the ferrous iron-salts medium. The first sub-culture without reduced sulphur achieved oxidation of at least 80% of the available ferrous iron (fig 3.7). The subsequent growth curve was directly comparable in rate and extent of oxidation for a culture growing in the presence of 1 mM tetrathionate (fig 3.7). Similarly 1 mgl^{-1} yeast extract as a supplement resulted in a slightly reduced lag phase in the growth curve for strain LM2, but could not extend the rate nor degree of iron oxidation beyond that of a non supplemented culture. The decline in these oxidation rates and the length of the lag phase were slightly reduced by growth in the presence of both 1 mM tetrathionate and 1 mgl^{-1} yeast extract together. This series of similar growth curves was contrasted with growth using either 1 mM glucose or 100 mgl^{-1} yeast extract as supplements. The glucose supplemented growth indicated little decline in oxidation rate prior to almost complete ferrous iron oxidation, although the lag phase initially was of a comparable length to that of the previous growth curves. The rate of oxidation in the presence of yeast extract (100 mgl^{-1}) was comparable with that of glucose, but the maximum rate was reached much quicker due to a greatly reduced lag phase (fig 3.7). However, the maximum rate declined quicker, prior to complete ferrous iron oxidation with 100 mgl^{-1} yeast extract than 1 mM glucose.

The physiological effects of nutritional supplements on strain LM2, in particular the apparent absence of effect of tetrathionate on growth, were not consistent through serial sub-culture. Ferrous iron grown cultures of strain LM2 could be maintained through continuous serial sub-culture only if suplemented with either 1 mM tetrathionate

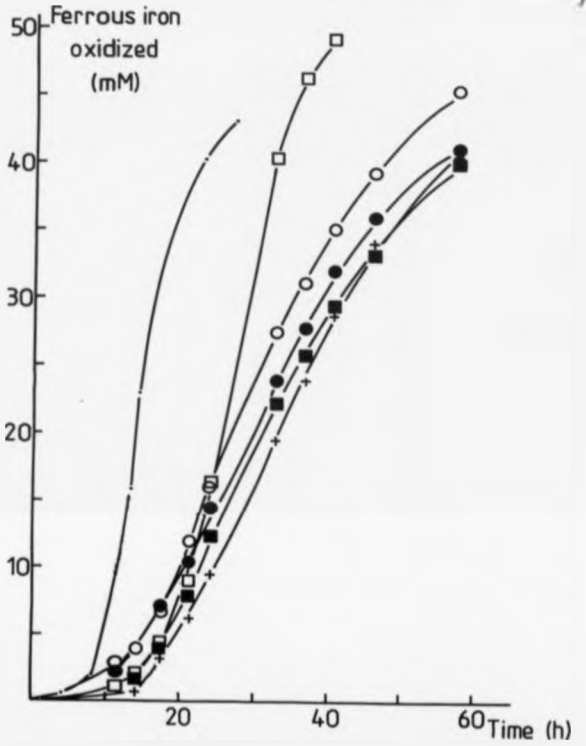


Fig 3.7
Batch growth curves of strain LM2 in 100ml ES at 50°C, pH 1.7 in shake flasks(250ml vol) at 120rpm with 50mM ferrous iron, only(+) and plus: 1mM tetrathionate(■), 1mgl^{-1} YE (●), 100mgl^{-1} YE(·)*, 1mM glucose(□), 1mgl^{-1} YE and 1mM tetrathionate(○). Inocula, 5%v/v were from 1mM tetrathionate supplemented culture. Flasks were trickled with 1%v/v CO_2 in air, except * - diffusion only.

or 100 mgl^{-1} yeast extract. Serial sub-cultures without supplementation or with 1 mM glucose declined in growth and eventually failed. Further, the pelletizing and washing of cell inocula before inoculation into the first sub-culture without supplement prevented growth. This suggested material carry over in the original inoculum as with strains TH1/8C1 (3.2.2). Having shown strain LM2 to grow in the continued presence of tetrathionate, washed cell inocula were used in a series of cultures with increasing concentrations of tetrathionate supplement (fig 3.8). The required concentration of tetrathionate for complete iron oxidation was small, 80% of available ferrous iron being oxidized in the presence of 1 µM tetrathionate. Once a concentration of tetrathionate, sufficient for complete oxidation was found (10 µM) increasing that concentration to excess, afforded little added advantage to the growth rate of the isolate (fig 3.8). In further investigating the concentration effect of tetrathionate at a range from 100 µM to the originally used 1 mM concentration (for unwashed cell inocula, from 0.1 mM tetrathionate supplemented cultures) the growth rate declined with increased initial tetrathionate concentration . Despite the rate decline all the cultures eventually went to completion (100% iron oxidized). That such relatively low levels of reduced sulphur were though required for complete oxidation (less than 10 µM tetrathionate) was consistent with reduced sulphur being an obligatory growth requirement but not an energy substrate, in these conditions. This requirement for reduced sulphur by strain LM2 could be sufficiently supplied by thiosulphate or flowers of sulphur (elemental sulphur) as alternative sources. In this experiment the use of solid sulphur created no apparent limitation in the growth of the organism, resulting in a growth rate similar to that observed with a thiosulphate (1.265 mM) supplement. These two growth curves differed only slightly in the length of the lag phase (fig 3.9). Being the first sub-culture the non-supplemented flask displayed complete oxidation, (due to carry over) but at a slower rate than with either elemental sulphur or thiosulphate.

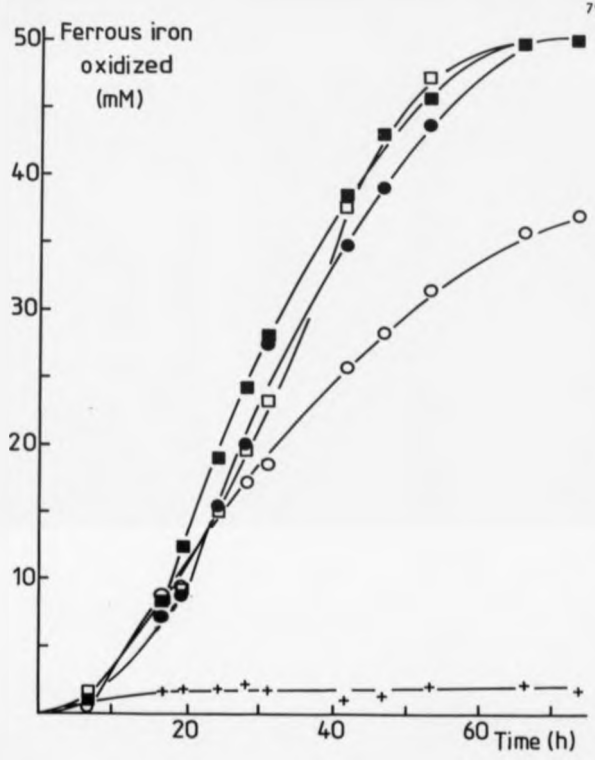


Fig 3.8
Effect of tetrathionate on batch growth of strain LM2. Shake flasks(250ml vol at 120rpm) contained 100 ml ES medium pH1.7 at 50°C with 50mM ferrous iron, only(+ , identical to sterile control, not shown) plus: 1μM(○), 10μM(●), 100μM (□) and 500μM(■) tetrathionate. Inocula, 3.5%v/v were washed by centrifugation prior to use. Flasks were trickled with 1%v/v CO_2 in air.

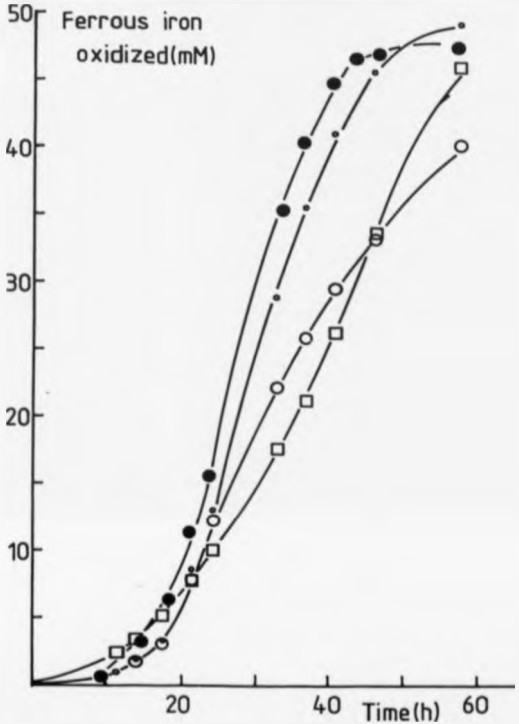


Fig 3.9
Comparison of batch growth curves of strain LM2 for different sources of reduced sulphur. Shake flasks(250ml vol shaken at 120rpm) at 30°C contained 100ml ES medium, pH1.7 with 50mM ferrous iron, gassed with 1%v/v CO_2 in air. Medium had no supplements (□), 100mgl^{-1} elemental sulphur flowers(•), 1mM tetrathionate (○) and 200mgl^{-1} thiosulphate (1.265 mM,●). Inocula were 5%v/v.

Both the longest lag phase and slowest growth rate were observed for the growth curve with 1 mM tetrathionate supplement (fig 3.9). This repeated the previous observation, in fig 3.7, that tetrathionate slightly increased the length of the lag phase in comparison to non-supplemented growth. (In that experiment, however, data points were lacking up to complete oxidation and hence the comparatively greater decline in maximum oxidation rate of the tetrathionate supplemented culture was not clearly illustrated). This confirmed the apparent inhibitory effect of excess tetrathionate, at least with respect to decline in oxidation rate, observed above. The requirement for reduced sulphur could not be replaced by glucose, which only enhanced oxidation in the presence of some residual reduced sulphur.

3.2.4 Strain ALV

In the first sub-culture into unsupplemented ferrous iron medium, strain ALV was capable of growth and complete ferrous iron oxidation (fig 3.10). The form of the resultant measured growth curve was not significantly affected by the presence of 1 mgl^{-1} yeast extract, other than a slight reduction in the length of the lag phase. However, the lag phase was more significantly reduced during growth in the presence of 1 mM tetrathionate. But, although the maximum oxidation rate was obtained more quickly and sustained longer for the tetrathionate supplemented growth curve it quickly declined when greater than 70% of the available ferrous iron had been oxidized (fig 3.10). Such rate decline was less marked but still evident when 1 mgl^{-1} yeast extract and 1 mM tetrathionate were present in the ferrous iron medium simultaneously. Associated with this complementation of supplements was a slight reduction in lag phase and a significantly quicker oxidation rate than those observed with 1 mM tetrathionate as the sole supplement (fig 3.10)

Separately, yeast extract at 100 mgl^{-1} and 1 mM glucose allowed complete and rapid oxidation. Both these culture conditions corresponded to the fastest observed oxidation rates for strain ALV in batch culture, but the reduction

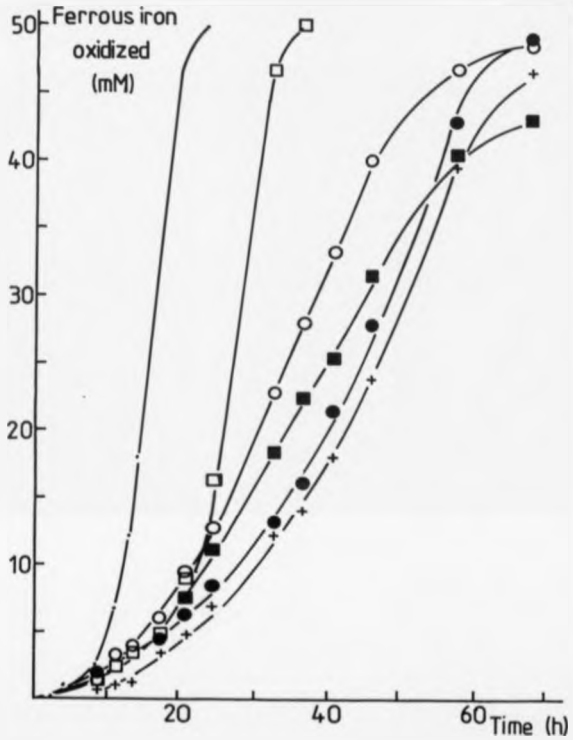


Fig 3.10
Batch growth curves of strain ALV in 100ml ES at 50°C. pH 1.7 in shake flasks(250ml vol) shaken at 120rpm with 50mM ferrous iron, aerated with 1%v/v CO_2 in air, except * - diffusion. Inocula. 5%v/v were from a non-supplemented culture. Medium additions were zero(+), 1mgl^{-1} YE(●), 100mgl^{-1} YE(·)*, 1mM tetrathionate(■), 1mM glucose(□), 1mgl^{-1} YE and 1mM tetrathionate(○).

in attendant lag phase was markedly greater with the yeast extract rather than the glucose supplement (fig 3.10). The level and rate of iron oxidation by strain ALV increased with increasing glucose concentration in the medium (fig 3.11). At a concentration of 0.01 mM, glucose had no effect on growth of strain ALV compared to it's capabilities in an unsupplemented medium. At 0.1 mM concentration a stimulated rate of oxidation was observed. A further increase in glucose concentration to 1 mM slightly reduced the length of lag phase and the onset of decline in oxidation rate, approaching 100% iron oxidized, but with little change in the actual maximum rate (fig 3.11). Therefore, supplementation of glucose at 1 mM was in excess for strain ALV, the requirement for growth under the conditions of these experiments lying between 0.01 mM and 0.1 mM glucose. (No investigation was made of more detailed values of glucose utilization - this was reported by Wood and Kelly, 1984, albeit in conditions where autotrophic iron oxidation without a source of reduced sulphur was not observed). Glucose supplemented iron grown cultures of strain ALV could though be maintained through serial sub-culture as could all the investigated culture conditions. Therefore, glucose stimulated ferrous iron oxidation in strain ALV without requirement for reduced sulphur (or any other organic supplement). Neither though was reduced sulphur apparently required for chemolithotrophic iron oxidation (in a carbon dioxide enriched atmosphere). Even with palletized and washed cell inocula, strain ALV grew in non supplemented iron-salts medium. Hence, as with the mesophiles, strain ALV oxidized iron with sulphate as the sole sulphur source. Though not requiring the presence of reduced sulphur and not being affected during growth by 1 mM tetrathionate, except in rate decline (fig 3.10, as with strain LM2, fig 3.9) iron oxidation by strain ALV was affected by the presence of thiosulphate (fig 3.12). With 200 mgl^{-1} sodium thiosulphate (1.265 mM) growth was incomplete, failing before oxidation of 60% of the available 50 mM ferrous iron. This apparent inhibition was alleviated by the presence of 1 mM glucose, but at the expense of a greatly

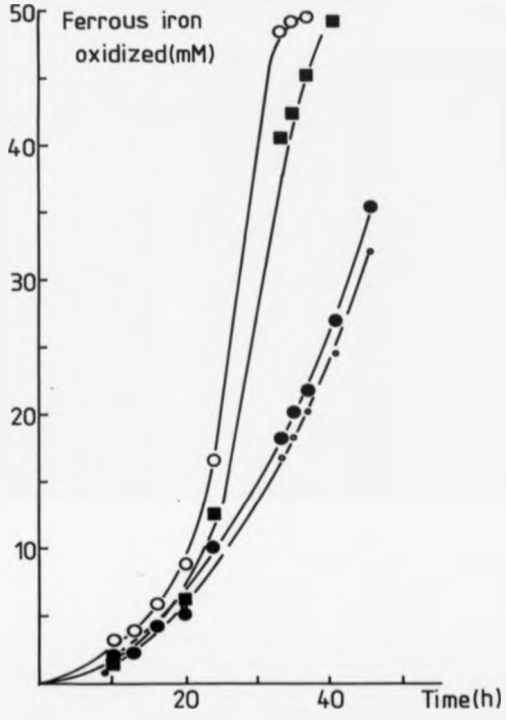


Fig 3.11
Effect of glucose concentration on batch growth curves of strain ALV. Shake flasks(250ml vol) at 120rpm contained 100ml ES medium, pH1.7, 50°C with 50mM ferrous iron and trickled with 1%v/v CO_2 in air. Inocula, 5%v/v were from a non-supplemented culture. Medium contained 0mM(∘), 0.01 mM(●), 0.1mM(■) and 1mM(○) glucose.

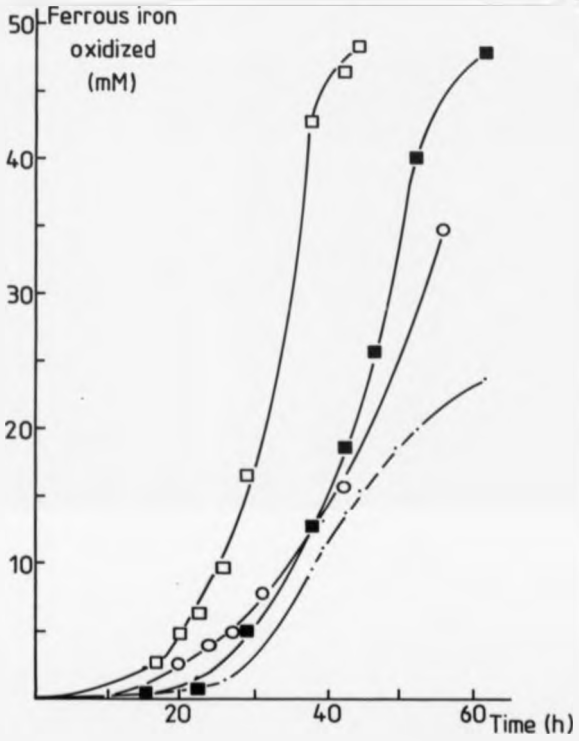


Fig 3.12
Inhibition in batch culture of strain ALV by thiosulphate. Medium(100ml), pH1.7 contained 50mM ferrous iron. Flasks(250ml vol) were shaken at 120rpm, 30°C and trickled with 1%v/v CO_2 in air. Inocula, 5%v/v were from non-supplemented culture. Additions were zero(○), 200mgl^{-1} thiosulphate(1.263 mM, •), 1mM glucose(□), 1mM glucose and 200mgl^{-1} thiosulphate(■).

increased lag phase compared to the growth of strain ALV in the presence of 1 mM glucose alone (fig 3.12). The length of the lag phases during batch growth increased with an increasing concentration of thiosulphate initially present, further supporting evidence of an inhibitory effect of thiosulphate (or a degradation product in the acid medium), on strain ALV.

3.3 EFFECT OF GROWTH SUPPLEMENTS - T.ferrooxidans and Sulfolobus BC65

To contrast the physiology of the moderate thermophiles both a mesophile and a thermophilic organism from the studied range of organisms (2.1) were tested with various supplemented ferrous iron media. T.ferrooxidans completely oxidised the available ferrous iron through serial sub-culture, without supplement, and did not require a carbon dioxide enriched atmosphere. No deviation in this growth pattern was apparent with the addition of 1 mM glucose (fig 3.13). Similarly, tetrathionate (1 mM) was ineffective in altering the rate or extent of ferrous iron oxidation, although it did incur a slight elongation in the length of the lag phase. However, thiosulphate appeared inhibitory to growth of T.ferrooxidans. Ultimately all the available ferrous iron was oxidised in the presence of either 0.5 mM or 1 mM thiosulphate, but after increased lag phases for the respective growth curves (fig 3.13), the length of increase, increasing with initial thiosulphate concentration.

Sulfolobus BC65 was not normally cultured in ferrous iron-salts medium. Therefore, shake flask cultures were inoculated from pyrite grown stock cultures into ferrous iron medium supplemented with 1 mM tetrathionate. Several serial sub-cultures were grown to remove any pyritic carry-over before such cultures were used for inoculating into experimental media. At 68°C, these cultures were prone to rapid ferric iron precipitation in the flasks, and cell lysis on the complete oxidation of ferrous iron.

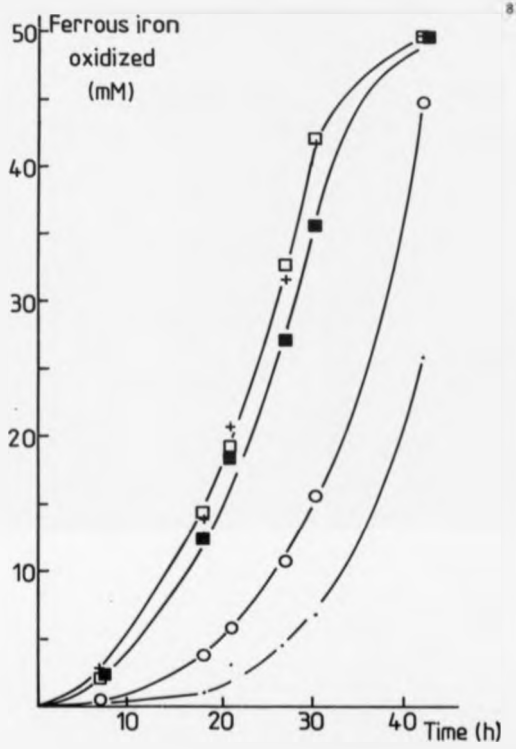


Fig 3.13
Batch growth curves of Thiobacillus ferrooxidans DSM 583 in 100ml ES medium, pH 1.7 with 50 mM ferrous iron shaken at 120rpm in flasks(250ml vol) at 30°C. Inoculated flasks, 5% v/v, contained no supplements(+), 1mM glucose(□), 1mM tetrathionate(■), 0.5mM(○) and 1mM(·) thiosulphate.

Therefore, the viability of an inoculum from a culture of greater than 80% ferrous iron oxidized could not be guaranteed. The first sub-culture into non-supplemented iron medium resulted in limited oxidation (fig 3.14). Not repeatable in a subsequent second sub-culture, such oxidation was probably due to carry-over with the inoculum. Spun and washed cells repeatedly did not grow in any medium and were therefore considered not viable under these conditions. So the effect of removing the supposed carry-over could not be shown. This limited level of oxidation in the first sub-culture was reduced in the presence of 1 mM glucose (fig 3.14) which had an apparently inhibitory effect on the oxidation by the Sulfolobus. (This apparent inhibition was not tested in conditions known to support complete growth of Sulfolobus BC65). Oxidation was completed (and sustained through sub-culture) with the addition of either thiosulphate or tetrathionate. Fig 3.14 shows the increased response of growth rate to the greater concentration of reduced sulphur present in 1 mM tetrathionate than in 1 mM thiosulphate.

3.4 PHYSIOLOGY OF STRAIN ALV

Strain ALV appeared to meet the criteria of an organism suitable for comparative iron oxidation studies with T.ferrooxidans (3.1). Principally the previously uncharacterized ability of this strain to chemolithotrophically oxidize ferrous iron in the absence of reduced sulphur (3.2.4) meant that it apparently differed from T.ferrooxidans only in the value of it's optimum growth temperature. Therefore, additional studies to further characterize the physiology of this iron oxidizer and to determine the exact optimum temperature for iron oxidation were carried out.

In shake flask experiments, and for the growth of stock cultures, the temperature of incubation reflected both the temperature at which cultures were originally isolated and that several isolates were incubated simultaneously

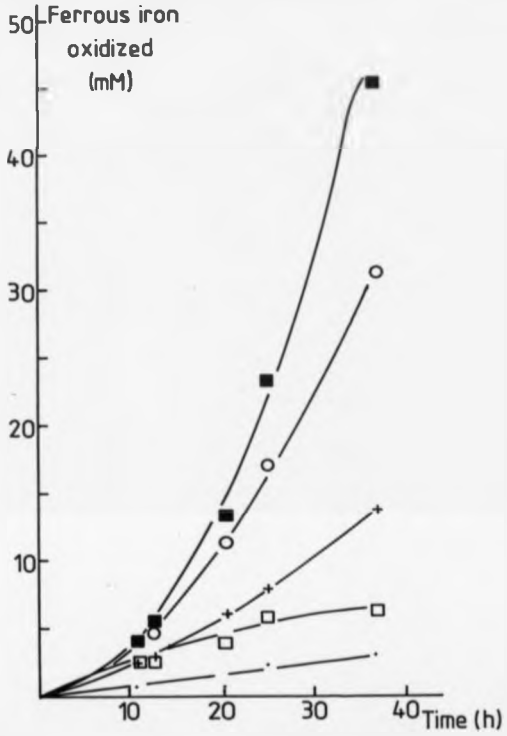


Fig 3.14
Ferrous iron oxidation during batch growth of Sulfolobus BC65. Inocula were 5%v/v from 1mM tetrathionate supplemented culture. Growth was in 100ml ES, pH1.7 at 68°C with 50mM ferrous iron in shake flasks(250ml vol) at 120rpm. Medium, trickled with 1%v/v CO_2 in air contained no supplements(+), 1mM glucose(□), 1mM thiosulphate(○), 1mM tetrathionate(■). Sterile control(·).

(2.2.2). Standard incubation at 30°C, 50°C and 68°C for mesophiles, moderate thermophiles and thermophile respectively, were not precise reflections of the optimum growth temperature of any one strain. Strains were grown at more precise temperatures during continuous culture (see 4.3).

3.4.1 Temperature for optimum iron oxidation

Batch growth curves for strain ALV were measured at temperatures from 35°C to 60°C inclusively at 5°C intervals. To ensure accurate incubation in comparable conditions, growth was in glass fermenter vessels, heated by circulating water pumps to plus or minus 0.5°C of required temperature (equipment - 2.2.2). The vessels were operated with a 300 ml volume of enriched salts at pH 1.7 with 50 mM ferrous iron but no nutritional supplements. During growth regular samples were taken and measured in duplicate for residual ferrous iron concentration. The averaged titration values, corrected for background (due to the end-point indicator), were converted to concentration values and plotted semi-logarithmically as mM ferrous iron oxidised against time. Growth failed at both 55°C and 60°C. Below these temperatures the measured rate curves exhibited increasing length of lag phase as the incubation temperature decreased . Oxidation was eventually taken to completion at 50°C, 45°C and 40°C and was still slowly continuing at 35°C after 62 hours. From the plots of initial rates, estimation was made of the doubling time of ferrous iron oxidation for strain ALV. As a plot of rate (doubling time) against temperature, this gave a temperature-growth curve indicating that the optimum growth temperature of strain ALV was closer to 45°C than 50°C (fig 3.15). (This plot could be compared with known data for some of the acidophilic iron oxidizers in this study,appendix 3). In contrast to the other moderately thermophilic strains ALV had a distinctly lower temperature optimum for autotrophic ferrous iron oxidation (though still significantly higher than for T.ferrooxidans).

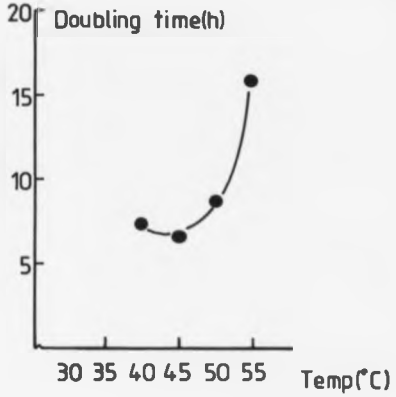


Fig 3.15
Temperature profile during batch growth on ferrous iron of strain ALV. Oxidation was measured in 300ml ES, pH1.7 with 50mM ferrous iron, contained in sterile 500ml glass fermenters. Magnetically stirred, vessels were inoculated with 7%v/v oxidised culture. Temperature of incubation (± 0.5°C), was 35°C, 40°C, 45°C, 50°C, 55°C and 60°C. Growth failed at the two higher temperatures. Residual ferrous iron was used to calculate % oxidation and plotted semi-logarithmically against time. Graph gives the relationship of growth rate (as doubling time of ferrous iron oxidation) calculated from these plots, against temperature of incubation. Vessels were aerated with 1%v/v CO_2 in air at 50mlmin^{-1}. (The optimum temperature for growth compared for other acidophiles, fig A3.3)

3.4.2 The pH limit for batch growth

To consider operating a continuous iron oxidation system of strain ALV under chemostat conditions, required that wall growth and other "natural" forms of biomass retention in the system could be prevented. To prevent the precipitation of iron and associated jarosites with related biomass build up (cf Bacfox process, Livesey-Goldblatt.et al 1977 and 4.3) strain ALV had to be capable of growth in conditions not conducive to precipitation ie in acid medium below the pH at which ferric iron precipitated. Growth of strain ALV was hence measured in batch flask culture with 50 mM ferrous iron, but no supplements, in enriched salts prepared at a range of initial pH values. (The measurement of pH and the associated use of buffers during measurement are outlined in 2.2.5). Growth was restricted by acidity in an experiment with initial pH values at 1.65, 1.38, 1.31, 1.26 and 1.21. The culture at pH 1.65 was a control, the organism having previously been grown successfully below this pH value. This range of pH values was intended to more closely indicate the "failure" point of iron oxidation relative to acidity of the medium. Plots were constructed of the rate of oxidation at each pH value (fig 3.16). Complete oxidation with slight decreases in rate due to increased acidity was measured at initial pH values of 1.65, 1.38 and 1.31. At pH 1.26 oxidation rate declined and growth failed whilst no oxidation was measured at pH 1.21. Strain ALV was hence shown to oxidize ferrous iron in medium both more acid than usual (pH 1.7) and more acid than that in which ferric iron would precipitate, the limit for complete oxidation of 50 mM ferrous iron being at or around pH 1.3.

3.4.3 Decline in oxidation rate

Routinely grown with 50 mM ferrous iron, strain ALV was tested for it's ability to grow with and oxidize 100 mM ferrous iron. Important in assessing possible limits of growth, during continuous culture, any effect on maximum oxidation rates had to be noted. In this respect the rate curves of fig 3.16 quickly deviated from the maximum linear rate. In parallel batch cultures, strain ALV completely oxidized both 50 mM and 100 mM ferrous iron.

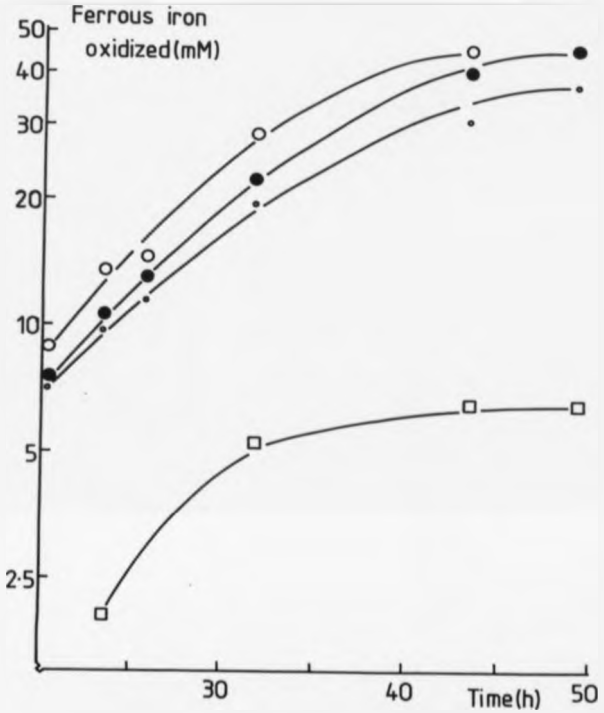


Fig 3.16
Rate plots of iron oxidised at different initial pH values during batch culture of strain ALV. Inocula, 5%v/v were from a culture at pH1.7. Growth in 100ml ES at 30°C with 1%v/v CO_2 in air was with 50mM ferrous iron, in shake flasks(250ml vol) at 120rpm , at initial pH values of pH1.65 (○), pH1.38(●), pH1.31(•), pH1.26(□) and pH1.21(no oxidation).

Though slower overall, the maximum rate was faster with 100 mM ferrous iron when measured as mM oxidized against time . This was with a slightly less severe decline in rate than with 50mM ferrous iron. When replotted as % ferrous iron oxidised, the rate curves were superimposable (fig 3.17). This indicated that deviation from the maximum rate of iron oxidation in batch cultures of strain ALV was related to the percentage of the available ferrous iron oxidised. (The relation of ferric iron to loss of maximum rate is discussed in section 4.2).

3.4.4 Contact inhibition with silicone rubber

Silicone rubber tubing was generally used in lab-scale continuous culture systems, for media transfer. This tubing had been shown as toxic to strain TH1 (Marsh 1985). As a precaution, silicone rubber tubing was placed in growing cultures of strain ALV. Found to be inhibitory to growth, an alternative tubing was tested and subsequently used. (Growth curves and discussion presented in 2.2.3).

3.5 SUMMARY

Of the strains in this study, strain TH3 was not capable of chemolithotrophic growth with ferrous iron, having an unknown essential growth requirement, present in yeast extract (3.2.1). That iron oxidation could be increased by 1 mM tetrathionate over the limited oxidation in the presence of $1 mgl^{-1}$ yeast extract (fig 3.1) suggested that it was a (minor) constituent from the yeast extract that was lacking and not a source of organic carbon. (Although carbon dioxide supplementation was not used with $100 mgl^{-1}$ yeast extract it's removal during growth with tetrathionate/yeast extract dual supplement was not investigated). Without the chemical identification of this growth requirement is whether as organic or inorganic, strain TH3 was only confirmed as obligately chemolithoheterotrophic, being incapable of chemolithotrophic or mixotrophic growth, under the conditions of this study. This lack of mixotrophy was only investigated with glucose which appeared directly

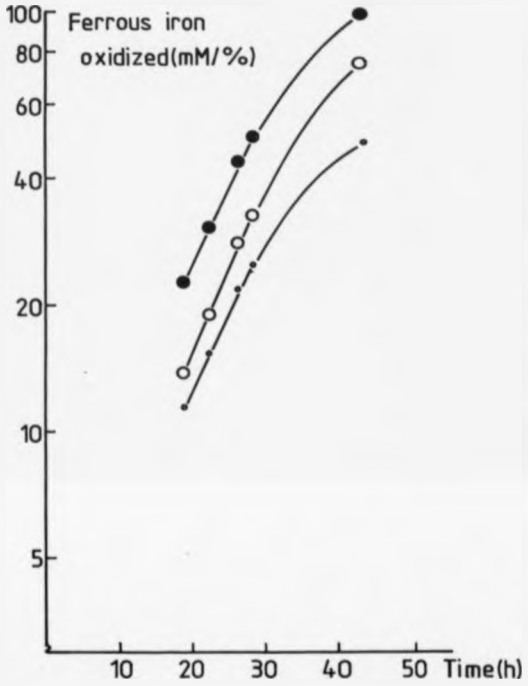


Fig 3.17
Comparison of iron oxidation rate as both % and mM oxidised against time in batch culture of strain ALV. Growth at 50°C was in 100ml ES at pH1.7 with 1%v/v CO_2 in air. Inocula were 5%v/v into flasks(250ml vol) shaken at 120rpm. Plots (on the same logarithmic scale) are for 50mM ferrous iron oxidised, mM(•) and as %(●) and for 100mM oxidised, mM and %(○). The rate plots are identical for % iron oxidised (except in the length of lag phase).

inhibitory of iron oxidation as did the presence of thiosulphate (fig 3.2). Strain TH3 was consequently routinely cultured with 100 ppm yeast extract supplement. In contrast, all the other four moderately thermophilic strains were shown capable of all three modes of nutrition, chemolithotrophic, chemolithoheterotrophic and mixotrophic (figs 3.3, 3.7, 3.10 and appendix 3). Displaying such growth, strains TH1 and BC1 were confirmed as sharing the same physiology (3.3.2). As with strain LM2, reduced sulphur was required during mixotrophic growth with glucose, although at concentrations below those needed to support oxidation during chemolithotrophic growth. The concentration of reduced sulphur for chemolithotrophic iron oxidation at less than 10 μM tetrathionate for strain LM2 (3.2.3) was at least an order of magnitude lower than for strain BC1/TH1 (3.2.2). Strain ALV had no reduced sulphur requirement, the first of these organisms to be shown capable of sulphate utilisation as sole sulphur source, in comparison with the mesophilic iron oxidisers (3.3) although the presence of reduced sulphur could reduce the lag phase during chemolithotrophic growth (fig 3.10). Tetrathionate could be replaced with thiosulphate or elemental sulphur for all the strains except strain ALV which displayed inhibited iron oxidation with thiosulphate (fig 3.12, cf strain TH3, fig 3.2). Such inhibition was concentration dependant and partially alleviated with glucose . That thiosulphate was unstable in acid medium meant that the inhibition may have been due to a breakdown product of thiosulphate, but that as other organisms still utilised this sulphur source in identical conditions, inhibition was still organism specific (irrespective of the specific compound involved). Growth was optimal for the moderate thermophiles during chemolithoheterotrophic growth but strains TH1, BC1 and LM2 were consequently routinely cultured with a 1 mM tetrathionate supplement despite this concentration of reduced sulphur being in excess. Strain ALV was consequently cultured in non-supplemented iron/salts media.

In contrast T.ferrooxidans DSM 583 was not mixotrophic

neither was rate stimulated by reduced sulphur although it was reduced by thiosulphate (fig 3.13). Thiosulphate was though a suitable source for Sulfolobus BC65 which while being non-mixotrophic was indeed, unlike T.ferrooxidans apparently inhibited by glucose (fig 3.14).

Therefore, strain ALV was capable of non supplemented iron oxidation, as was T.ferrooxidans, but at a growth optimum of 45°C as opposed to 32°C for T.ferrooxidans (appendix 3). It was also capable of oxidizing 100 mM ferrous iron the decline in rate being apparently linked to the percentage ferrous iron oxidized and therefore to the ferrous to ferric iron ratio (3.4.3). Such oxidation was possible at pH values at which ferric iron would remain in solution but was totally inhibited by an unidentified consituent from flexible silicone rubber tubing. This toxicity was not observed for T.ferrooxidans. Therefore during chemolithotrophic growth on ferrous iron strain ALV displayed the closest nutritional similarity to T.ferrooxidans, and presented the climax in a progressive decline in the requirements of the moderate thermophiles for reduced sulphur ie strains TH1/BC1, strain LM2 and strain ALV in order of significant decreasing need for reduced sulphur during chemolithotrophic growth.

4 IRON OXIDATION KINETICS - APPARENT K_m AND INFLUENCE OF FERRIC IRON ON GROWTH

4.1 INTRODUCTION

The physiology and culture conditions for the acidophilic iron oxidizers were more fully determined (Chapter 3). The affinity of each strain for ferrous iron substrate was chosen as an indicator of their potential capabilities. Cell suspensions had previously been used in estimating values of K_m in iron oxidation (1.7.1,1.8.1,1.8.2). This was investigated as a system capable of summarizing the ability of different organisms to oxidize iron. Such a system had to generate values that: (1) provided comparable measurements for any acidophile, (2) gave organism specific values, (3) were capable of explaining the growth curve generated during iron oxidation and (4) could be used as a predictive tool for organism performance eg during continuous iron oxidation and/or in the presence of ferric iron.

4.2 IRON OXIDATION KINETICS - NON GROWING CELL SUSPENSIONS

4.2.1 Establishing experimental conditions

It had to be shown that thermophilic isolates could provide active (iron oxidizing) cell suspensions in standardized experimental conditions. These had to display rate values proportional to cell concentration in order to apply Michaelis-Menten kinetics.

The strains were cultured under the chemolithotrophic conditions, as defined in 3.5. Strain TH1 was supplemented with and without yeast extract (0.01% w/v) as a comparison with strain TH3. *Sulfolobus* BC65 was cultured on pyrite to reduce problems of handling (3.3). Cells were

harvested by centrifugation and washed at pH 1.7 to remove residual iron (conditions of growth, media and harvesting-2.3.1,2.4).

The effect of pH was investigated for the mesophiles. Kelly and Jones (1978) reported that hydrogen ions (pH 1.2) were a non competitive inhibitor of ferrous iron oxidation by T.ferrooxidans. Therefore cell suspensions of T.ferrooxidans were prepared at pH values of 1.37, 1.74 and 1.95. A double reciprocal Lineweaver-Burke plot of oxidation rate in the oxygen electrode indicated a competitive form of inhibition at low pH (fig 4.1). The values of apparent K_m, 1.11 mM, 1.30 mM and 2.12 mM, increased with acidity but the V_{max} was consistently estimated at 890 n moles oxygen per min per mg protein. Two experiments with L.ferrooxidans did not indicate any trend in inhibition by hydrogen ions. Due to this (partial) influence of hydrogen ions the pH was carefully maintained at pH 1.7 (the pH during growth experiments. The influence of pH on iron oxidation was not further investigated, although strain ALV was known to be inhibited during batch growth ,3.4.2). The conclusions for the data from these experiments were hence limited to conditions creating a pH value of 1.7.

All the cell suspensions oxidized iron, with only Sulfolobus BC65 displaying auto-respiration in the absence of substrate. The activity of all the strains decreased on storage depending on strain and temperature. Cells were added cold to the oxygen electrode in order to preserve activity during the experiments. Significant activity loss was though displayed by L.ferrooxidans and more severely with strain TH1. This percentage loss over time was measured (fig 4.2) and used to correct rate values during each experiment.

Acceleration phases appeared prior to the onset of the maximum (linear) rate during iron oxidation in the oxygen electrode. The length of delay was less at low substrate concentrations but increased on the addition of ferric iron. This was typically of the order of three minutes (maximum) for the mesophiles and Sulfolobus BC65. However,

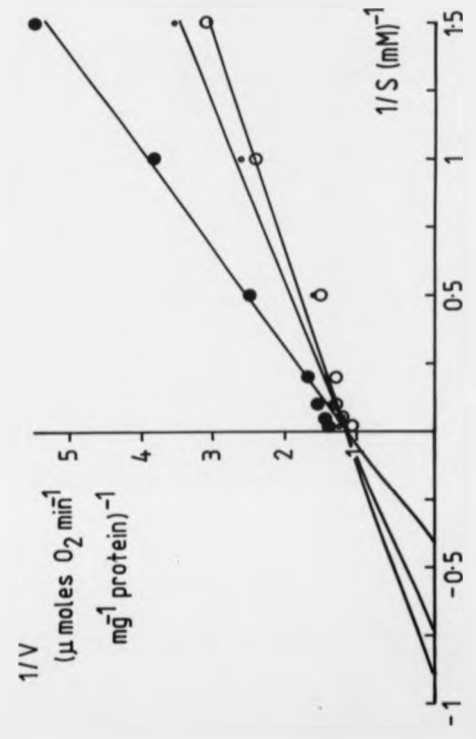


Fig 4.1
Double reciprocal plot for oxygen uptake rates of cell suspensions of T.ferrooxidans at different pH values. Each plot was the average of two rate determinations at each ferrous iron substrate concentration at 30°C. The linear relationships were calculated by regression analysis. The pH values were pH1.37(● ; r=0.997; K_m=2.12 mM), pH1.74(• ; r=0.998; K_m=1.30mM), pH1.95(○ ; r=0.997; K_m=1.16mM). Indicative of competitive inhibition, the shared V_{max} value was 890 n moles oxygen per min per mg bacterial protein.

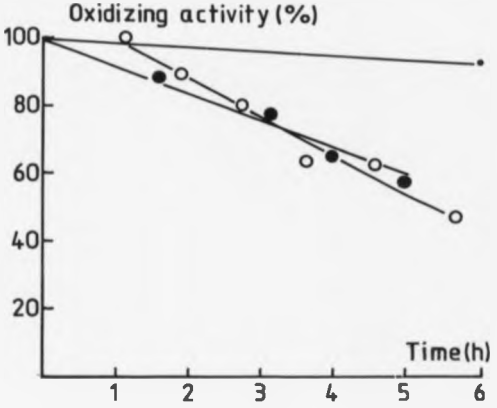


Fig 4.2
Loss over time of iron oxidizing activity in freshly harvested cell suspensions of L.ferrooxidans(●) and strain TH1(○) for a typical experiment. Oxidation rate of the initial substrate concentration (10mM or 50mM ferrous iron) in an oxygen electrode was repeated at intervals during the experiments. Constructed for each experiment these graphs of rate decline were used to compensate all measured values for the effect of activity loss. Typical percentage loss over the same time scale with a T.ferrooxidans suspension(•) is shown in comparison.

neither the fresh cells of strain TH1 nor of strain TH3 exhibited any acceleration phase in the substrate range used, even on the addition of ferric iron (10 mM). (This counteracted the possibility that they were lag phases generated on adding room temperature cells to heated reaction chambers). In contrast strain ALV had short acceleration phases but rapidly deviated from the maximum oxidation rate prior to either substrate or oxygen exhaustion. This also occurred during oxygen uptake in a Warburg manometer (2.4.3). The plot of uptake over time was a curve although the rate increased with increased cell (protein) concentration (fig 4.3). The oxygen uptake for both strains TH1 and TH3 was linear over time until approaching substrate exhaustion (fig 4.4). In an effort to induce deviation from linear oxidation, both strains TH1 and TH3 were measured for oxidation of 50 mM ferrous iron in the presence and absence of 50 mM ferric iron. Little response in the rate of oxidation was observed (fig 4.4-for strain TH1). The non linear uptake of strain ALV was therefore strain specific and not an artefact of the oxygen electrode.

Oxidation rate was proportional to cell concentration for all the organisms, although the specific values could alter between experiments. These relationships (fig 4.5) were used to set the limits of cell concentration for each organism. Within these limits data was mathematically processed to provide standard kinetic values. These were generated graphically, and by several methods, to offset errors inherent in any one form (2.4.2). (Fig 4.6, alternative graphical representations of the data for one experiment with T.ferrooxidans).

4.2.2 T.ferrooxidans

Calculated from double reciprocal plots the apparent K_m was 1.34 $\pm$ 0.16 mM ferrous iron, for six separate determinations with cell suspensions of T.ferrooxidans. Each determination used freshly harvested cells. The cell concentration used during these experiments ranged from 12.7 to 93.3 µg protein per ml final reaction volume. The calculated V_{max} values ranged from 0.885 to 2.941 µ moles

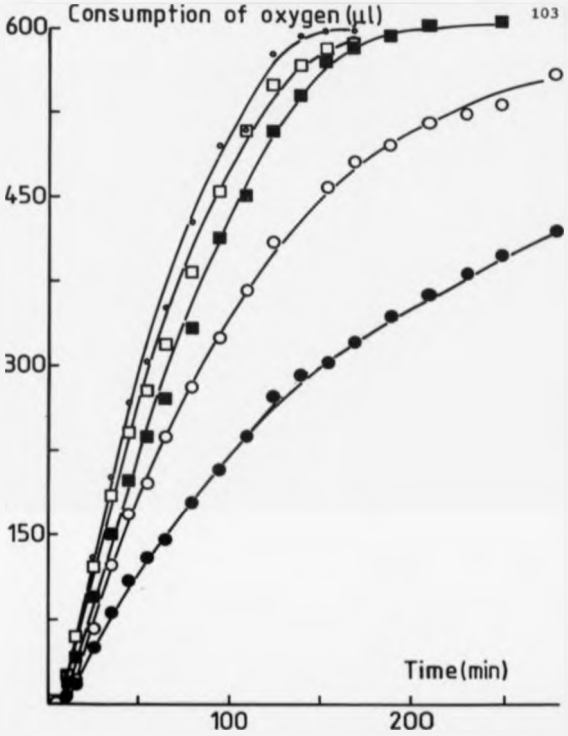


Fig 4.3
Oxygen consumption over time during iron oxidation by a cell suspension of strain ALV measured in a Warburg respirometer. Warburg bath at 45°C was shaken at 120 strokes per min. Total reaction volume was 2.5ml, at pH1.7 containing 50mM ferrous iron. Oxidation was initiated by tipping cell suspensions of different protein concentration from flask side-arms. Rate curves (corrected for atmospheric pressure changes) were measured for final cell concentrations (µg protein per ml of reaction vol) of 14.7(●), 29.3(○), 58.6 (■), 87.9(□) and 117.2(•).

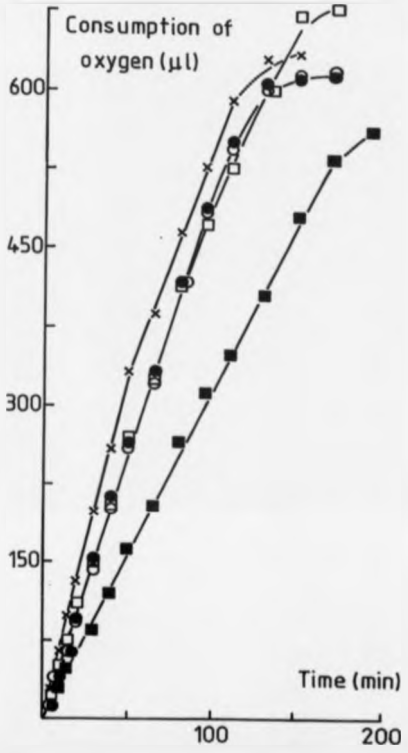


Fig 4.4
Comparison of oxygen uptake by cell suspensions of strains TH1 and TH3. Side-arm flasks attached to manometers were placed in a Warburg bath, at 45°C, shaken at 120 strokes per min. Oxidation started on flushing cell suspensions from the side arms (final volume 2.5ml). Each flask contained 50mM ferrous iron. Oxidation by strain TH1 was measured with(○) and without(●) added 50mM ferric iron for a protein concentration of 222 µg protein per ml reaction vol. Oxidation of 50mM ferrous iron by strain TH3 was measured at protein concentrations of 51(■), 152(□) and 455(×) µg protein per ml reaction vol.

oxygen per min per mg protein, an average of 1.66 ± 0.77 μ moles oxygen per min per mg protein. The value of V_{max} appeared to drop with an increase in the protein concentration used (table 4.1).

TABLE 4.1

Values for apparent K_m for cell suspensions of T.ferrooxidans measured for ferrous iron oxidation in six separate experiments and calculated from Lineweaver-Burke plots. The corresponding protein concentrations used (μg per ml) and V_{max} estimations (μ moles oxygen per min per mg protein) are shown (K_m is in mM).

K_m	1.30	1.30	1.51	1.10	1.49	1.37
V_{max}	2.74	2.13	1.66	1.33	1.02	0.88
protein conc	12.7	33.7	37.7	60.0	76.0	93.3

This inverse relationship between protein concentration and calculated V_{max} contradicted that observed with actual rate measurements (fig 4.5). These values were noted to be chronological in their order of measurement over a two year period. In one of the experiments the V_{max} was 1.25 and 1.33 μ moles oxygen per min per mg protein for respective protein concentrations of 36 and 60 μg per ml. This appeared to confirm the influence of time, but was not further investigated. (The estimations of apparent K_m were 1.09 mM and 1.10 mM but the first value was calculated from single rate determinations and was not included in the averaged data).

Alternative graphical representation of the data as S/V against S (2.4.2) gave the mean apparent K_m for the same six determinations as 1.44 ± 0.61 mM ferrous iron. The mean from the corresponding V against V/S plots was 1.27 ± 0.16 mM. The three graphical presentations therefore, yielded a close agreement in the average value but with different degrees of standard deviation.

Graphical plots were also used in determining the form of

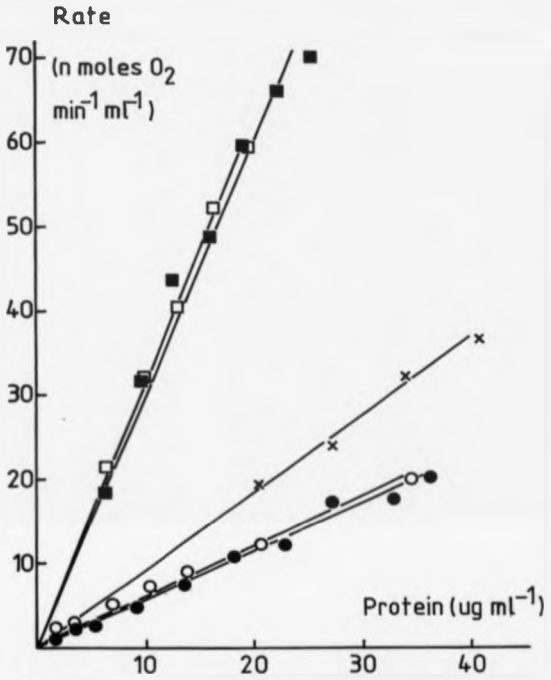


Fig 4.3
Relationship of oxygen uptake in the oxygen electrode (as n moles oxygen consumed per min per ml reaction vol) to cell concentration (as ug protein per ml reaction vol). Each rate was measured in duplicate at the appropriate temperature in a total reaction vol of 3ml. Substrate concentration was 50mM ferrous iron. Linear plots were calculated by linear regression analysis for T.ferrooxidans(r=0.991, ■), strain ALV(r=0.997, ○), strain TH1(r=0.998, ×), strain TH3(r=0.998, □) and Sulfolobus BC65(r=0.996, ●)

inhibition with 10mM ferric iron (fig 4.6). Apparently competitive in nature each plot was never precisely as expected in theory. For the experiment of fig 4.6 there was a consensus in the average values of V_{max} of 1.02 and 1.06 μ moles oxygen per min per mg protein, in the absence and presence of ferric iron from the three graphical forms. This observation was consistent throughout and was taken as indicitive of competitive inhibition.

With only minor differences observed in presenting inhibition data, the double reciprocal Lineweaver-Burke plot was used almost exclusively to represent the form and degree of inhibition in all the strains. (Unless indicated, subsequent individual values of both apparent K_m and K_i in the text were calculated from the appropriate double reciprocal plot).

With T.ferrooxidans. in the presence of 10 mM ferric iron, values for K_p (the K_m during inhibition) were generated from four experiments ranging from 6.2 mM to 7.8 mM. (In fig 4.6 K_p was 6.61 mM, 5.79 mM or 6.40 mM depending on the plot). The value of K_p increased with increased inhibitor concentrations of 25 mM and 100 mM ferric iron to 16.7 mM and 40.2 mM respectively. Directly calculating the inhibitor constant K_i from the K_p values (2.4.2) for 10 mM ferric iron gave values from 1.16 mM to 3.83 mM ferric iron. Alternatively, using the values of the slopes from the appropriate double reciprocal plots for both inhibited and non inhibited iron oxidation, the calculated K_i range was 1.43 mM to 4 mM. (The more common expression of inhibition, K_i, was subsequently used throughout this study though the value varied with the method of calculation, as above, 2.4.2). For direct comparison with all the strains, K_i calculations from the slopes on graphical plots were used throughout.

The K_i determinations were spread from 1.43 mM to 4 mM ferric iron, but in these particular experiments the corresponding apparent K_m values demonstrated a similar spread. As apparent K_m increased in value so the corresponding value of K_i increased (table 4.2). Although the values of either K_i or apparent K_m appeared

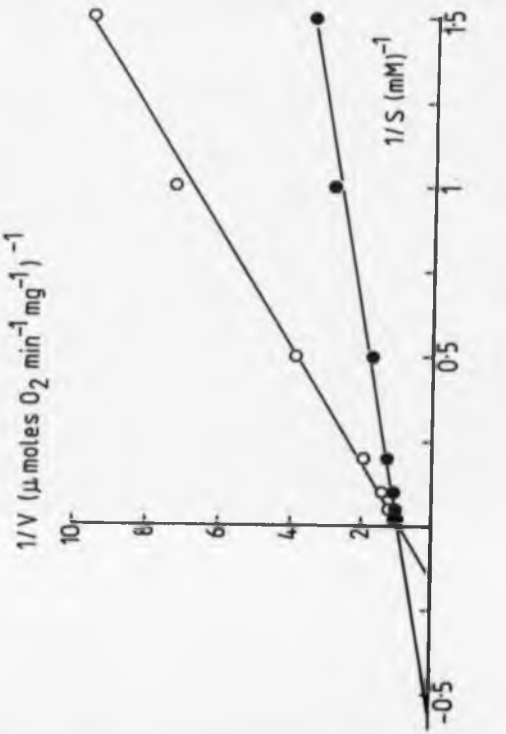


Fig 4.6
Graphical presentation of alternative linear forms of the Michaelis-Menten equation for iron oxidation by a cell suspension of T.ferrooxidans. Oxidation rates for ferrous iron were in duplicate; plotted as the average value(●) at 30°C, and initiated by adding cells at a final concentration of 81.8 μg protein per ml. Single rate measurements of oxidation were measured in the presence of 10mM ferric iron(○). Linear plots were calculated from regression analysis. Fig 4.6a. Double reciprocal plot. The K_s was 1.76mM. The V_{max} was 1.047 μ moles oxygen per min per mg protein. Correlation coefficient, r, was 0.996. Inhibited by 10mM ferric iron, K_s was 6.61mM, V_{max} 1.124 μ moles oxygen per min per mg protein. The calculated K_i was 3.63mM, with r 0.998.

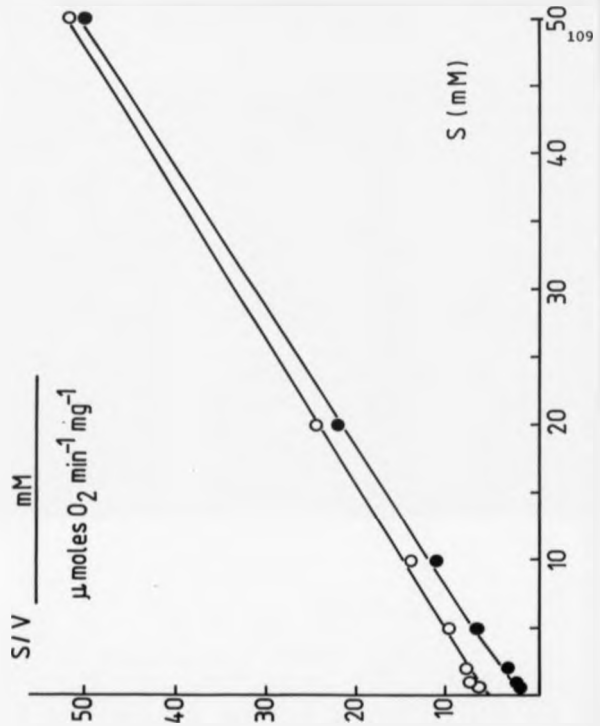


Fig 4.6b
Plot of S/V against S (Woolf plot) for T.ferrooxidans cell suspension, from the same experimental data of Fig 4.6a. Value for K_m(for iron oxidation, ●) was 1.69mM, the V_{max} (reciprocal value of the slope; r=1) was 0.971 µ moles oxygen per min per mg protein. With 10mM ferric iron present during oxidation(○), K_p was 5.79mM and V_{max}, 0.940 µ moles oxygen per min per mg protein. Correlation coefficient was 0.999, K_I 4.12mM.

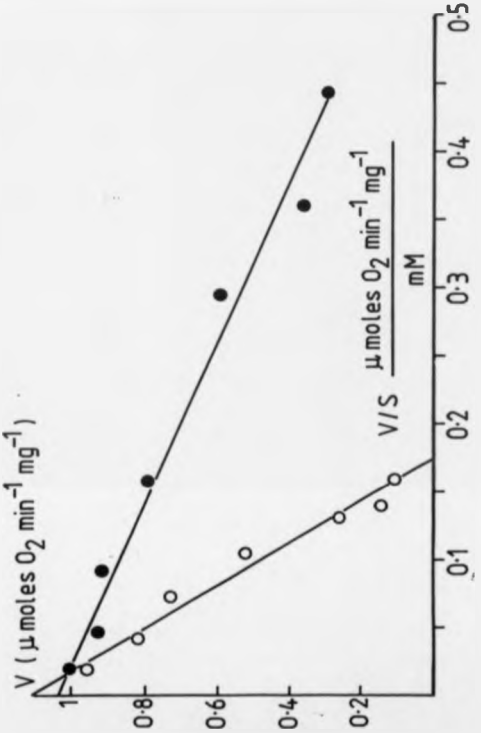


Fig 4.6c
Presentation of V against V/S (Eadie-Hofstee) plot for iron oxidation by T.ferrooxidans cell suspension (cf figs 4.6a,b). The apparent K_m (for iron oxidation, ● r=-0.990) was 1.71mM; V_{max} was 1.038 μ moles oxygen per min per mg protein. With 10mM ferric iron initially present during oxidation(○), K_p was 6.40 mM, V_{max} 1.113 μ moles oxygen per min per mg protein and K_i 3.65 mM; r was -0.983.

inconsistent, for these four particular experiments the ratio of both measured constants, K_i to K_m was itself almost constant.

TABLE 4.2

Inhibitor constant K_i measured for the effect of 10 mM ferric iron on iron oxidation by cell suspensions of T.ferrooxidans. Calculated from the slopes of double reciprocal plots from four separate experiments the K_i values (mM) are presented in relationship to the corresponding apparent K_m (mM) measured during the same experiments.

K_m App	0.61	1.30	1.37	1.71
K_i	1.43	3.04	3.87	4.0
K_i/K_m	2.34	2.34	2.80	2.34
K_i (from K_p)	1.16	2.02	2.83	3.83
K_i/K_m	1.90	1.55	2.07	2.24

If a relationship between both constants was valid this would support the validity of apparent K_m values that fall outside the calculated mean figure, eg 0.61 mM in table 4.2 and the mean of 1.34 $\pm$ 0.16 mM (for double reciprocal plots). The evidence of table 4.2 suggested a relationship of ferrous and ferric iron to the cells in terms of the concentrations that elicited a given physiological response (for ferrous iron, the concentration (K_m) at which oxidation functioned at half the maximal rate, V_{max}). If the individual constants were related irrespective of the actual values then variations in experimental values could have been of microbial origin. That is, the spread of experimental values would have been considered due to experimental error. If however, the spread of values still appeared to have a set form of relationship then it was likely that it was not the physical part of the experiment but the biological, causing the variation. Hence an inherent relationship would not be changed by the physical conditions that altered specific values. This included methods of

calculation for K_i. Alternative values still increased with increasing K_m (table 4.2). The mean values for the ratios (with standard deviation of n-1) were 2.45 $\pm$ 0.29 and 1.94 $\pm$ 0.29. Therefore the determinative method affected changes in the "value" of the relationship, it did not affect the principle of a "fixed" correlation between K_m and K_i.

Strain LA was known to be in a different homology group from the above DSM 583 (Harrison,1984;1.7.3). In two experiments, the apparent K_m was 0.56 mM and 1.14 mM ferrous iron with 32.3 and 35.4 µg protein per ml final reaction volume, respectively. The respective V_{max} determinations were 1.98 and 1.82 µ moles oxygen per min per mg protein. Using 10 mM ferric iron the attendant K_i values for both cell suspensions were 1.55 and 3.1 mM. Taking the ratios of K_i to K_m gave values of 2.72 and 2.77, further evidence of a fixed relationship between these two constants. Both Thiobacillus strains were measured together. The similarities in kinetic constants did suggest some influence from experimental variation in conditions (table 4.3). The only apparent variable between the two determinations was the degree of oxidation in the batch cultures used. In the first determination both cultures were 95% oxidized, in the second between 75% and 85% oxidized. The other determinations with T.ferrooxidans were with cultures approaching the higher level of oxidation and therefore any correlation was speculative (not being investigated). In the final assessment the kinetic values were indistinguishable for these two strains from these determinations (with the possible exception of the K_i/K_m ratio).

4.2.3 L.ferrooxidans

Eight determinations of apparent K_m for non-growing cell suspensions of L.ferrooxidans gave a mean estimation from double reciprocal plots of 0.25 $\pm$ 0.08 mM ferrous iron. Protein concentrations used in these determinations ranged from 61.8 to 155.2 µg per ml. The calculated V_{max} values, from 0.351 to 0.747 µ moles oxygen per min per mg protein gave a mean value

TABLE 4.3

Comparison of kinetic measurements made for cell suspensions of two variants of T.ferrooxidans, strains LA and DSM 583, for two experiments in which each strain was compared in identical conditions. Apparent K_m is in mM ferrous iron, K_i mM ferric iron, protein concentration is µg per ml and V_{max} µ moles oxygen per min per mg protein.

		K_m	K_i	[protein]	V_{max}	K_i/K_m
expt 1	strain LA	1.14	3.10	35.4	1.82	2.72
	DSM 583	1.30	3.04	33.7	2.13	2.34
expt 2	strain LA	0.56	1.55	32.3	1.98	2.77
	DSM 583	0.61	1.43	29.8	1.8	2.34

of 0.493 ± 0.125 µ moles oxygen per min per mg protein. The only significant deviation from these recorded V_{max} values was for one determination after 24 h storage of the cell suspension at 6°C. With an apparent K_m of 0.16 mM ferrous iron, V_{max} was 2.14 µ moles oxygen per min per mg protein. As no determination was made for this suspension on the day of harvesting, a repeat experiment was performed measuring both apparent K_m and V_{max} directly after harvesting and after 24 h storage. Repeat experiments did not show significant rate rises after cell storage.

The apparent K_m values from the graphical variants of plotting V against V/S and S/V against V, for the same data, were 0.26 ± 0.08 mM and 0.15 ± 0.07 mM respectively. These closely averaged results for the eight determinations were measured over three years. Though the individual values of V_{max} varied, the spread of values showed no obvious correlation with the chronology of determination, the concentration of protein used nor the degree of oxidation in the cultures prior to harvesting the cell suspensions. Nonetheless, over the spread of experimental data, even after rates had been corrected both for different protein values used, and the loss of activity during the actual experiments, the specific

oxidation rates for each substrate concentration indicated a continuous and consistent downward trend over the range of determinations. This apparent loss of oxidative capacity, without affecting the apparent K_m values was indicative that substrate affinity and rate of substrate oxidation were under separate control. In the absence of obvious causation such rate loss could be suggested as a simple degradative loss in this progressively sub-cultured lab strain of L.ferrooxidans. although it was noted (above) that no trend in V_{max} values could be associated with these apparently decreasing oxidation rates.

In one out of two experiments using 10 mM ferric iron, there was no significant effect on oxidation rate or apparent K_m of L.ferrooxidans. In the second experiment the changes in rate were relatively small, the data being more reliant than most of the experiments on statistical analysis to the effect that inhibition was present (fig 4.7). The form of the double reciprocal plot was suggestive of competitive inhibition and the V_{max} values with and without ferric iron present were 0.515 and 0.503 μ moles oxygen per min per mg protein respectively. The change in K_m of 0.294 mM to a K_p of 0.380 mM was such that the calculated K_i constant was 37.9 mM ferric iron. In a separate determination the use of 25 mM ferric iron had a more definite inhibitory effect on iron oxidation but without significant change in the estimate of K_i. With the apparent K_m estimated for this experiment as 0.360 mM, the K_i was 38.2 mM ferric iron. With the change to a different ferric iron concentration there was little significance in comparing the K_i to K_m ratio (not least being only 2 results). However, the ratios with 10 mM and 25 mM ferric iron were 128.9 and 106.1.

Strain 8C was from the group of Leptospirillum-like bacteria (1.8.1). Using 90.6 μg protein per ml, the apparent K_m for one determination was 0.308 mM ferrous iron, whilst V_{max} was 0.202 μ moles oxygen per min per mg protein. Physiologically, strain 8C displayed a growth rate as a doubling time typically of 29 h compared to the 11 h doubling time of L.ferrooxidans (Harrison,1982) but in this single determination both strains were comparable

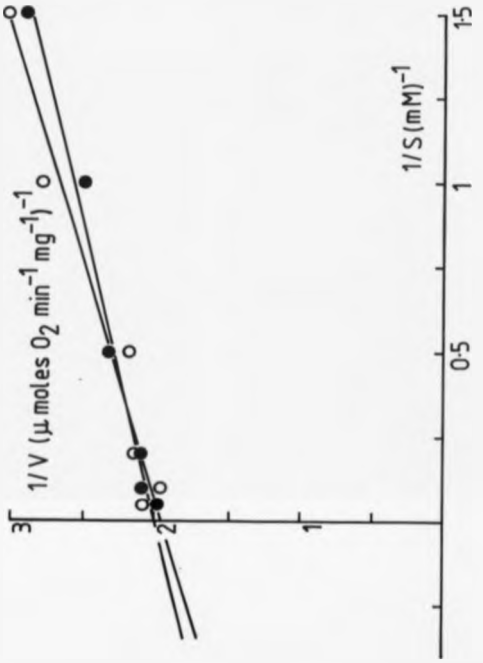


Fig 4.7
Double reciprocal plot of oxygen uptake rate against substrate concentration for cell suspension of L.ferrooxidans at pH1.7 and 30°C. Cells were used in the oxygen electrode at a final concentration of 107.3 μg protein per ml . Iron oxidation was measured in the absence(●) and presence(○) of 10mM ferric iron intially. The apparent K_m was 0.294mM with a V_{max} of 0.503 μ moles oxygen per min per mg protein; correlation coefficient r was 0.990. During inhibition, K_p was 0.380mM and V_{max}, 0.515 μ moles oxygen per min per mg protein. Calculated K_i was 37.9mM ferric iron; r was 0.974.

in apparent K_m value. This result indicated that growth rate of an organism was probably not an indicator of apparent K_m for a suspension, or vice versa under the relevant experimental conditions.

4.2.4 Moderate thermophiles

Strain TH1, grown autotrophically provided four determinations of apparent K_m with a mean value of 1.03 ± 0.086 mM ferrous iron. Grown chemolithoheterotrophically (with a 0.01% w/v yeast extract supplement) a single determination for apparent K_m was 1.015 mM ferrous iron. Therefore, the previous physiological growth conditions of the cells did not affect the value of apparent K_m measured for non-growing cells, in the oxygen electrode. Cell yields were increased in the presence of the yeast extract (effect on growth rate, 3.2.2) but for all five determinations, the cell concentration in the oxygen electrode was between 31 µg and 41 µg per ml protein. The V_{max} values, for the autotrophically grown cells ranged between 1.032 and 2.137 µ moles oxygen per min per mg protein, an average of 1.57 ± 0.48 µ moles oxygen per min per mg protein. The yeast extract supplemented cells gave a V_{max} value of 1.54 µ moles oxygen per min per mg protein.

The estimations of apparent K_m, from the alternative graphical plots for strain TH1 did show significant differences. From the S/V against S plots the mean value for the four separate determinations was 2.05 ± 0.63 mM ferrous iron and from V against V/S plots the mean value was 1.06 ± 0.06 mM ferrous iron. Variations in values also occurred during the estimations of K_i. Iron oxidation was inhibited by 10 mM ferric iron. Two K_i values were 2.59 mM and 2.78 mM for autotrophically grown and yeast extract supplemented cells, respectively. The form of the inhibition was ostensibly competitive in nature (shown for autotrophically grown cells, fig 4.8), though the values of V_{max} were consistently lower in the presence of 10 mM ferric iron, by all three methods. (The kinetic data for the experiment in fig 4.8 is summarized in table 4.4). In table 4.4 values of K_i were calculated

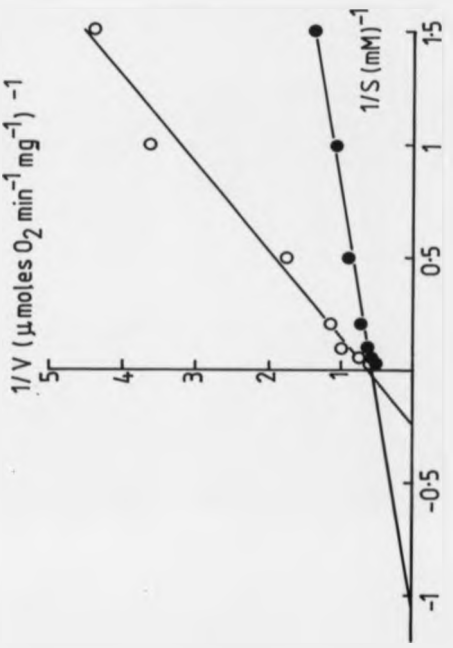


Fig 4.8
Double reciprocal plot for oxygen consumption rate at changing ferrous iron concentration with(○) and without(●) 10mM ferric iron added for cell suspension of strain TH1. Using autotrophically grown cells, oxidation was measured in the oxygen electrode at 50°C and at pH1.7. Oxidation initiated with a final cell concentration of 31.5 µg protein per ml. Apparent K_m was 0.95mM, V_{max} 1.77 µ moles oxygen per min per mg protein; correlation coefficient r was 0.989. In the presence of ferric iron, K_i was 2.59mM (3.01mM calculated from K_D value) V_{max} was 1.38 µ moles oxygen per min per mg protein and r was 0.991. The graphical form was indicative of competitive inhibition by ferric iron.

directly from K_p in order to be comparable between the three graphical forms. As noted above, using the values of the slopes for the double reciprocal plot of this experiment (fig 4.9) the K_i was 2.59 mM (a K_i/K_m ratio of 2.73). In contrast, two separate experiments yielded comparable K_i values of 3.67 mM and 5.43 mM (ratios of 3.42 and 5.43 respectively). Therefore, as with T.ferrooxidans K_i values varied but with no observed consensus in K_i/K_m ratios.

TABLE 4.4

Comparison of the kinetic data for a cell suspension of strain TH1 calculated from alternative linear plots. Values of K_i were calculated from the relationship of K_m to K_p, all three values being in mM. Values for V_{max} are in μ moles oxygen per min per mg protein. Determinations 1-3 are in order respectively of double reciprocal plot, S/V against S and V against V/S.

	K_m	V_{max}	K_p	V_{max} (inh)	K_i
1	0.95	1.77	4.11	1.58	3.01
2	2.01	2.04	5.66	1.82	5.49
3	1.02	1.81	4.24	1.63	3.16

With strain TH3, measured in comparison with strain TH1, mean values of apparent K_m were 0.45 $\pm$ 0.11 mM and 0.45 $\pm$ 0.13 mM ferrous iron from double reciprocal and V against V/S plots respectively. Calculated from four separate determinations, the average value using the S/V against S plot was 0.62 mM but for extreme values of 0.12 mM to 1.49 mM ferrous iron. As with strain TH1 the S/V against S plot consistently disagreed with the close results of the other two plots. With the double reciprocal plot the values for three V_{max} values were broadly similar (for protein concentrations of 20.3 to 63.7 μg per ml) at 3.15, 3.70 and 4.83 μ moles oxygen per min per mg protein. The fourth value was 0.96 μ moles oxygen per min per mg protein. There was no obvious reason for this comparatively low value. This determination was

repeated after 24 h storage with the same cells but fresh solutions. The V_{max} value had decreased, but the value of apparent K_m had increased, a phenomenon repeated after prolonged storage at 6°C. (Although rate measurements were only determined singly, the data is summarized in table 4.5).

TABLE 4.5

Values of apparent K_m and V_{max} for a cell suspension of strain TH3 and their change over time. Cell concentration was 63.17 μg per ml protein in the oxygen electrode, storage was at 6°C. Time in days, K_m in mM and V_{max} in μ moles oxygen per min per mg protein.

Storage time	Apparent K_m	V_{max}
0	0.35	0.96
1	0.76	0.85
1*	0.81	0.82
18	1.40	0.74

* Measured with half the previous cell concentration

No trend was observed in the degree by which values changed over time (in subsequent experiments) but in three out of four suspensions V_{max} decreased in value over time and for all four the value of apparent K_m increased in value overall, after storage.

In the presence of 10 mM ferric iron, the K_i for strain TH3 from four experiments averaged at 1.89 ± 0.39 mM ferric iron. The competitive form of inhibition was always indicated (fig 4.9). In the experiment of fig 4.9 the values of apparent K_m of 0.47 mM and K_i of 1.56 mM gave a K_i/K_m ratio of 3.32. This value was part of a range of ratios, 2.70, 3.32, 5.0 and 5.70 that as with strain TH1 showed no consistency in value. Neither was there any trend between the spread of apparent K_m values and the spread of K_i values. Despite this, in one experiment (using 13 μg per ml protein) an extreme measurement was made for apparent K_m of 0.077 mM ferrous iron. This experiment was not included in the averaged data, but with

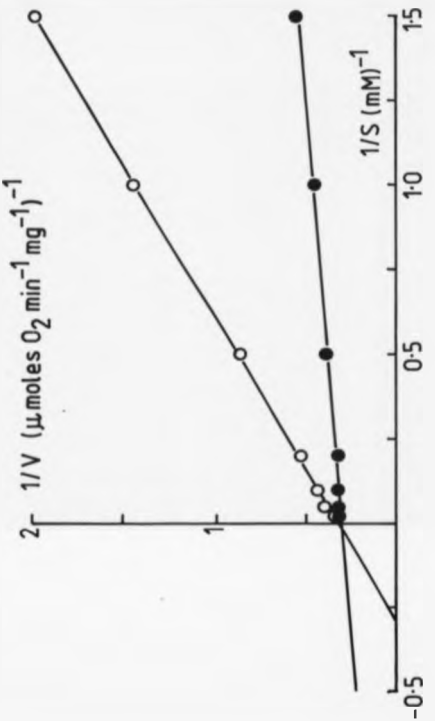


Fig 4.9
Double reciprocal plot for oxygen uptake during iron oxidation at changing substrate concentration by cell suspension of strain TH3. Measured at 30°C and pH1.7 in an oxygen electrode oxidation was with(○) and without(●) added 10mM ferric iron. Final cell concentration was 20.3 μg protein per ml. Apparent K_m was 0.47mM, V_{max} 3.16 μ moles oxygen per min per mg protein. Correlation coefficient r was 0.994. During inhibited iron oxidation, K_m was 3.32mM. The calculated K_i was 1.56mM, V_{max} 3.03 μ moles oxygen per min per mg protein; r was 0.999.

a V_{max} value of 4.48 μ moles oxygen per min per mg protein was comparable in the estimate of maximum rate. With a corresponding K_i value of 0.284 mM ferric iron, the ratio of K_i/K_m was 3.69. This was close to the average value of the above ratios, 4.18. Though not repeatable the causation of this extremely low apparent K_m value had a corresponding effect on ferric iron inhibition such that despite the relatively low values of K_i and K_m the relationship between them (and indeed the value of V_{max}) were comparable to those above, again indicating a possible (organism specific) link between these two constants, irrespective of specific experimental values.

The standard deviation on the estimated mean values for apparent K_m was relatively high for strain ALV. Measured over six experiments, the data was formed from rates taken from quickly changing rate curves (4.2.1). Respectively, from double reciprocal, V against V/S and S/V against S plots the calculated mean values of apparent K_m were 2.96 ± 0.68 mM, 3.06 ± 0.80 mM and 3.56 ± 0.73 mM. As with these mean values, the cell concentrations used during these experiments were relatively consistent at 42.5 ± 4.45 μg per ml protein. This was also true of the determinations of V_{max}. Using the double reciprocal plots the V_{max}, from four experiments was 0.66 ± 0.04 μ moles oxygen per min per mg. The other two values were 0.84 and 0.94 μ moles oxygen per min per mg, an overall mean of 0.74 ± 0.13 μ moles oxygen per min per mg protein.

Ferric iron (10 mM) inhibited iron oxidation rate by strain ALV, but competitive inhibition was only indicated in limited conditions (fig 4.10). The general form of the inhibited oxidation plot was that of a curve. The plots at the higher substrate concentrations, plus ferric iron (5, 10, 20 and 50 mM) consistently formed a statistically competent linear relationship. Due to the high reproducibility of the visual spacing of such inhibited rates in five experiments, the probability of a linear relationship, at all seven substrate concentrations used, was rejected. The mathematical generation of linear relationships for the restricted plots (in fig 4.10 the correlation coefficient for four points, r, was 0.995) in

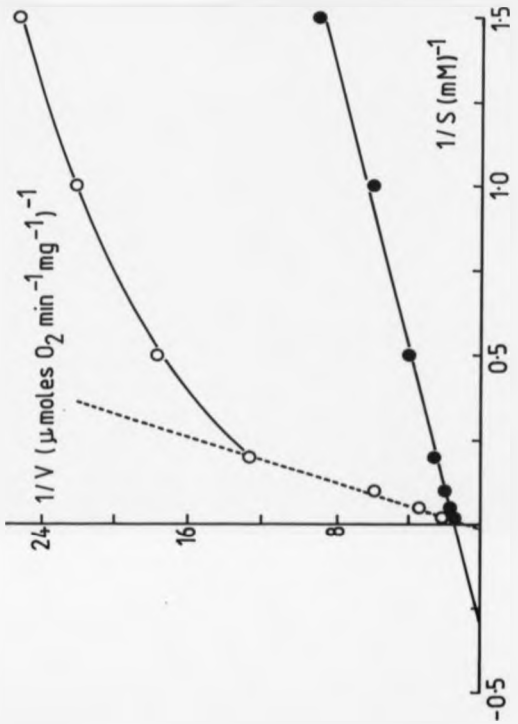


Fig 4.10
Lineweaver-Burke plot of oxygen consumption at changing ferrous iron substrate concentration for a cell suspension of strain ALV. Oxidation was measured at 45°C and pH1.7 with 35.5 μg protein per ml. In the absence of ferric iron(●) the linear relationship (r,0.998) estimated apparent K_s at 3.28mM and V_{max} at 0.674 μ moles oxygen per min per mg protein. With 10mM ferric iron(○) the data plots were formed into a curve. Utilising the plots at the higher substrate concentration this relationship was linear (hatched line, r-0.995, extended to show contrast with overall curve). Using this secondary plot, K_s was calculated as 0.91mM (K_m, 92.5mM) and V_{max} as 1.58 μ moles oxygen per min per mg protein.

all the experiments prevented this being an arbitrary manipulation of data. The assumption of competitive inhibition could be made (albeit in limited circumstances). Accepting the limitations and inherent error in using such plots, under the assumption of competitive inhibition by 10 mM ferric iron, over the substrate range of 5 mM to 50 mM ferrous iron, the calculated K_i values were averaged at 1.02 $\pm$ 0.12 mM ferric iron (for five determinations). The corresponding apparent K_m values had the largest spread of any of the tested organisms of 2.13 mM to 4.11 mM, yet the value of K_i appeared closely related to these. The mean of the five individual K_i to K_m ratios was 0.34 $\pm$ 0.07. Such an observation was problematic for strain ALV in that did it (1) provide additional support to the ratio of K_i to K_m being a fixed, organism specific value, or did it (2) by providing a result supported by (coincidental) precedance (eg T.ferrooxidans. 4.2.3 and strain TH3, above) justify what was in effect an erroneous manipulation of the data? As mentioned above and illustrated in fig 4.10 the data points consistently formed linear relationships that curved off due to the plotted rates at low substrate concentration. On consideration it seemed that point (1) above was more correct the corollary of which was (as suggested in point (2)) that having shown a fixed ratio of K_i to K_m in itself justified the manipulation of the data. This in turn supported the contention of iron oxidation in strain ALV being affected by mixed forms of inhibition in the presence of ferric iron.

Strain LM2 was not comprehensively studied, and no measurement was made of ferric iron inhibition of iron oxidation. In one single determination with a cell concentration of 44.5 μg per ml protein, apparent K_m was 1.525 mM. The V_{max} value was 0.58 μ moles oxygen per min per mg protein (the associated correlation coefficient r, was 0.997). By the alternative methods of S/V against S and V against V/S plots the apparent K_m determination was 1.97 mM and 1.54 mM respectively.

4.2.5 *Sulfolobus* BC65

Kinetic evaluation of iron oxidation by *Sulfolobus* BC65 resulted in a mean apparent K_m, measured by double reciprocal plots, from three pyrite grown cultures of 0.56 ± 0.03 mM ferrous iron. The alternative plots estimated apparent K_m as 0.60 ± 0.03 mM (V against V/S plot) and 0.695 ± 0.29 mM (S against S/V plot). The protein concentration used was between 16.5 µg and 33.6 µg per ml resulting in an averaged value for the V_{max} as 1.34 ± 0.26 µ moles oxygen per min per mg protein. A fourth determination of apparent K_m was made using chalcopyrite grown cells (continuously sub-cultured on a copper based substrate). With 17 µg per ml protein the apparent K_m and V_{max} values were 0.59 mM ferrous iron and 1.29 µ moles oxygen per min per mg protein.

Competitive inhibition of oxidation was indicated, in the presence of 10 mM ferric iron, but only under conditions comparable to that of strain ALV (fig 4.11; cf fig 4.10). In the experiment of fig 4.11 the linear relationship of five inhibited rate points had a relatively low correlation coefficient, r of 0.98 but this was also true for the non-inhibited oxidation line. This was a feature of *Sulfolobus* experiments and may have been in part caused by difficulty in obtaining original rate values from noisy chart recorder traces (in turn, probably attributable to the temperature at which the oxygen electrode system was operated). Despite this V_{max} values from fig 4.11 at 1.11 and 1.13 µ moles oxygen per min per mg protein were a strong indicator of competitive inhibition. From this the K_i value was calculated as 1.71 mM ferric iron. Two further estimations of K_i were made using 25 mM ferric iron. The corresponding K_i values (calculated as for fig 4.11) of 1.28 mM and 1.94 mM, in their spread, did not indicate any change in inhibitor constant due to the increase in inhibitor concentration used. The ratios of the K_i to K_m values, in the order as above, were 2.87, 2.33 and 4.22.

4.2.6 Inhibitors of ferrous iron oxidation

Concentrations of copper in solution (as hydrated copper sulphate) in the oxygen electrode inhibited iron oxidation

Fig 4.11
Double reciprocal plot of oxygen uptake during ferrous iron oxidation at changing substrate concentration by a cell suspension of Sulfolobus BC65. Oxidation was measured in the oxygen electrode, at 68°C and pH1.7. Oxidation was initiated with cells at a final concentration of 33.6 ug protein per ml. In the absence of inhibitor(● ,r, 0.98) apparent K_m was 0.61mM, V_{max} 1.11 μ moles oxygen per min per mg protein. With 10mM ferric iron present, oxidation rate was inhibited(○), the subsequent plots only partially fitting a linear pattern (hatched line,r, 0.98) curving off at low substrate concentration (complete line). Based on the linear relationship, inhibition was competitive (K_m 4.21mM) with K_i equal to 1.71mM and V_{max} 1.13 μ moles oxygen per min per mg protein.

by cell suspensions of T.ferrooxidans. The form of inhibition appeared close to being competitive(fig 4.12). Assuming this the K_i (with 50mM copper) was estimated at 54.5 mM copper (K_p 1.52 mM), a K_i to K_m ratio of 75.7. The difference in this experiment between V_{max} values was 0.89 and 0.97 μ moles oxygen per min per mg protein, in the absence and presence of copper respectively. In the experiment of fig 4.12 a concentration of 200 mM copper produced a K_i of 178.7 mM copper. For this determination the ratio of K_i to K_m was 127. In comparison, 200 mM copper produced a K_i of 178.2 mM copper in the genetically distinct Thiobacillus LA strain, but the specific ratio of K_i to K_m was 318.2.

With L.ferrooxidans copper was an uncompetitive inhibitor of iron oxidation (fig 4.13). Though poorly defined by linear relationships, the inhibited and non-inhibited data plots were clearly separate with no obvious point of interface for the associated linear plots (fig 4.13). The values of V_{max} of 0.50, 0.61 and 0.50 μ moles oxygen per min per mg protein were reduced by the 200mM copper to 0.255, 0.26 and 0.25 μ moles oxygen per min per mg protein. This was from the respective S/V against S, double reciprocal and V/S against V plots. With lack of detailed experimental data, an estimation of K_i value could not be made.

In attempting to measure copper inhibition of Sulfolobus BC65 cells at 65°C, the presence of copper in the oxygen electrode caused a chemical oxygen demand, not observed at 30°C. Incorporating suitable controls, measurement of oxidation rates were often erratic and no definitive value of K_i was obtained. However, through several test experiments of varied experimental conditions, the form of inhibition was consistently estimated as being competitive in nature (within the confines of error present in these observations).

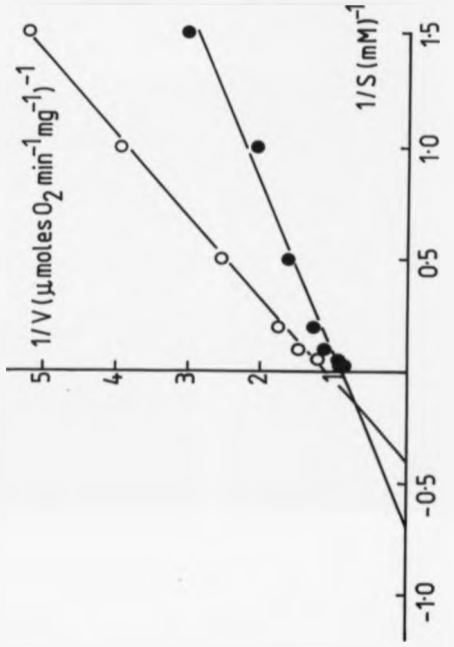


Fig 4.12
Double reciprocal plot of oxygen consumption during ferrous iron oxidation at changing substrate concentration in the presence(○) and absence(●) of 200mM copper by a T.ferrooxidans cell suspension. Measured at 30°C, pH1.7 with 93.3 µg protein per ml in the absence of copper apparent K_m, for this experiment was 1.41mM and V_{max} 1.08 µ moles oxygen per min per mg protein. The correlation coefficient r was 0.991. The estimated K_i was 178.7 mM copper (K_m, 2.76 mM) with a V_{max} of 0.913 µ moles oxygen per min per mg protein. For the linear relationship of inhibited oxidation, r was 0.997.

Fig 4.13
Double reciprocal plot of oxygen consumption during ferrous iron oxidation at changing substrate concentration in the presence(○) and absence(●) of 200mM copper by a *i.ferrooxidans* cell suspension. Oxidation was measured in the oxygen electrode at 30°C, pH1.7 with 120 µg protein per ml. Apparent K_s was 0.21mM, V_{max} 0.50 µ moles oxygen per min per mg protein. Correlation coefficient r was 0.629. In the presence of copper inhibited oxidation gave estimated K_s as 0.13mM, V_{max} 0.255 µ moles oxygen per min per mg protein; r was 0.647.

All the tested acidophilic strains differed in growth rate during batch culture. This was influenced by growth conditions, particularly nutritional supplements (Chapter 3). Kelly and Jones (1978) showed that in batch culture the principal limiting growth factor of T.ferrooxidans was ferric iron product inhibition of ferrous iron oxidation rate. Batch growth was used in this study to test the response of growing cultures to ferric iron and the connection if any to estimations of K_i for ferric iron on cell suspensions.

Batch cultures were grown with upto 25mM ferrous iron, in shake flasks at pH 1.8. Ferric iron supplements were used at a range of final concentrations. As a result of the pH control of ferric iron stock solutions, oxidation rates were also measured in the presence of 62.5 mM sodium (without ferric iron) in control flasks (2.2.1). All the oxidation curves were plotted semi-logarithmically as iron oxidized against time.

4.3.1 Rate curves-mesophiles

The growth response to ferric iron was compared for both T.ferrooxidans strains, strains LA and DSM 583. Iron oxidation rates were comparable for both strains in all three tested culture conditions, when plotted as iron oxidized (mM) against time (fig 4.14). Estimating the doubling time (from the concentration of iron oxidized), the growth rates, μ, on 25 mM ferrous iron, for strain DSM 583 and strain LA were 0.154 per h (h^{-1}) and 0.149 h^{-1} respectively. Strain LA exhibited the longer lag phase (fig 4.14). This was preserved in the presence of 62.5 mM sodium, the resultant rate curves being almost duplicate determinations for each strain. The sodium ion was regarded as negligible on any subsequent response of ferric iron to both growth and oxidation rate.

In the initial presence of 125 mM ferric iron, the growth rate, μ, was 0.095 h^{-1} for both strains but with a greater increase in the length of lag phase for strain DSM 583. Overall, the oxidation rates for both strains were similar with and without ferric iron present (fig 4.14), all the cultures eventually going to completion. The effect of

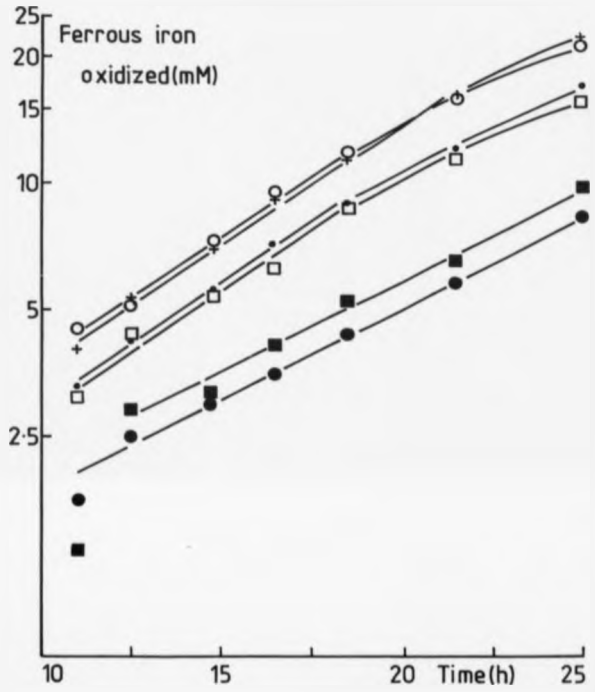


Fig 4.14
Effect of ferric iron on logarithmic growth curves, as iron oxidized against time, for batch cultures of T.ferrooxidans, strain DSM 583(○,+,●) and strain LA(•,□,■). Cultures (100ml) were in ES pH1.8, shaken at 120rpm and 30°C(in 250ml flasks) with 25mM ferrous iron: only(○,□); plus 62.5mM sodium(+,•); plus 62.5mM sodium and 125mM ferric iron (●,■). Inocula were 5%v/v from oxidized cultures grown with 50mM ferrous iron.

ferric iron was concentration dependent for strain DSM 583 (fig 4.15). Grown with 20.2 mM ferrous iron and a range of ferric iron concentrations of 12.1 mM, 32 mM, 72.9 mM and 121.5 mM ferric iron, growth was measured in the increasing presence of ferric iron as 0.112, 0.133, 0.124, 0.098 and 0.089 h^{-1} (fig 4.15). All the rate determinations went to completion but both reduction in rate and increase in length of lag phase only appeared at the higher concentrations of ferric iron, 72.9 mM and 121.5 mM.

Grown with 25 mM ferrous iron L.ferrooxidans gave a growth rate, of 0.092 h^{-1}. The almost linear rate plot displayed only a slight decline in rate as oxidation reached completion (fig 4.16, cf fig 4.15). However, data plots for the parallel culture with an additional 125 mM ferric iron present were superimposable indicating no apparent effect of this concentration on iron oxidation during batch culture. Neither then, by implication did the sodium present (62.5 mM) have any influence on ferrous iron oxidation. The oxidation rate was then measured for L.ferrooxidans at a range of ferrous iron concentrations (5 mM, 10 mM, 20 mM, 30 mM and 40 mM) in order to investigate effect of substrate concentration on rate and the possibility of measuring apparent K_s in batch culture. Doubling time and therefore μ could not though be clearly distinguished from the linear rate plots. There was no trend in oxidation rate with increasing substrate concentration. Neither was there a relationship between the length of lag phase (which were identical) and substrate concentration. On this basis an estimation of K_s in batch growth was not achieved.

4.3.2 Rate curves-moderate thermophiles

The responses of strains TH1 and TH3 were measured in comparison with 100 ppm yeast extract supplement in both cultures. Both strains had marginally reduced lag phases as the substrate concentration increased, over the range of 5mM to 50mM ferrous iron but with little alteration in the observed doubling times. Growth was exponential with strain TH1 declining from the maximum oxidation rate

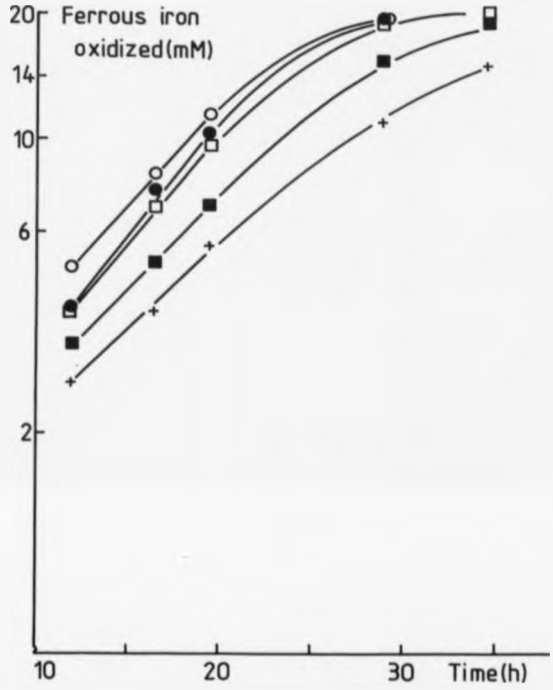


Fig 4.15
Growth curves of T.ferrooxidans DSM 583 showing the effect of increasing initial ferric iron concentration on ferrous iron substrate oxidation. Flasks(250ml vol) were shaken at 120rpm at 30°C and inoculated, 5%v/v. Medium was ES pH1.8 and ferrous iron concentration was 20.2mM ferrous iron with added ferric iron concentrations of 0mM(○), 12.1mM(●), 32mM(□), 72.9mM(■) and 121.5mM(+) ferric iron.

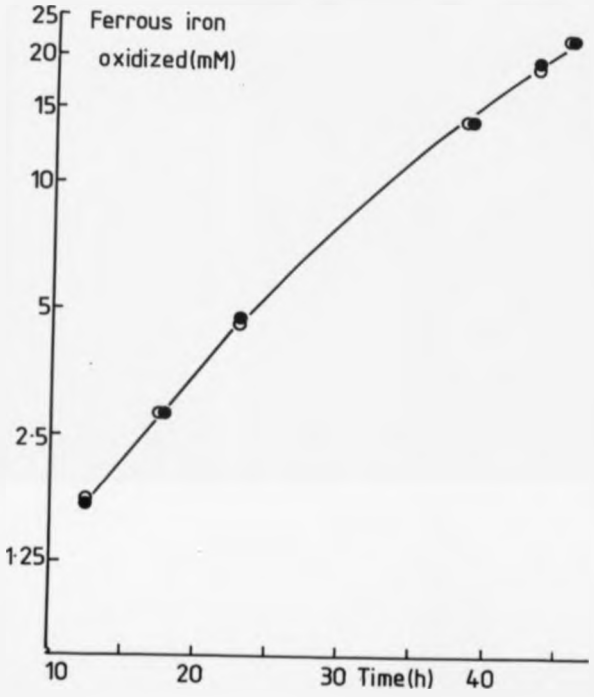


Fig 4.16
Logarithmic plot of iron oxidised during batch culture of L.ferrooxidans. Shake flasks(250ml vol) were inoculated 5%v/v and shaken at 120rpm at 30°C. Medium was 100ml ES at pH1.8. Oxidation of 25mM ferrous iron was measured in the presence(●) and absence(○) of an initial concentration of 125mM ferric iron.

earlier in the growth curve than observed for strain TH3 (fig 4.17). The total oxidation time was effectively doubled by 125 mM ferric iron for both strains. The presence of 62.5 mM sodium resulted in a very slightly reduced lag phase for strain TH3 but did not alter the form of the growth curve for strain TH1.

Inhibition of growth by ferric iron was concentration dependant for both strains but different in it's effects. Plotted as logarithmic rate curves the oxidation rate for 20.2 mM ferrous iron progressively declined with increasing additional ferric iron from 12.1 mM to 121.5 mM for strain TH1 (fig 4.18). The length of the lag phase increased and the maximum rate declined earlier in growth as the ferric iron concentration increased. In comparison, with strain TH3 the addition of ferric iron resulted in an increase in lag phase with 12.1 mM and 32 mM ferric iron but with an additional dramatic decrease in oxidation rate with 72.9 mM and 121.5 mM ferric iron (fig 4.19). With strain TH1 the absence of yeast extract during autotrophic growth increased the length of the lag phase but did not alter the influence of ferric iron.

The initial rate of iron oxidation increased with substrate concentrations of 5 mM to 50 mM ferrous iron for strain ALV (fig 4.20). From these curving plots estimates of doubling time decreased from 5.9 h to 2.5 h. Converted to the reciprocal growth rate, μ a double reciprocal plot estimated apparent K_m as 8.07 mM with μ (V_{max}) of 0.31 h^{-1} ie a doubling time during maximum iron oxidation in batch culture of 2.26 h.

Strain ALV was sensitive to ferric iron during growth. With 25 mM ferrous iron and 121.5 mM ferric iron in the medium, no growth was observed either for autotrophic conditions, or with a 0.01% w/v yeast extract supplement. Oxidation rates were not influenced by 62.5 mM sodium. In yeast extract supplemented media with 20.2 mM ferrous iron, complete oxidation was possible in the presence of 12.1 mM and 32 mM ferric iron. Particularly at the latter concentration the subsequent rate curve was increased in

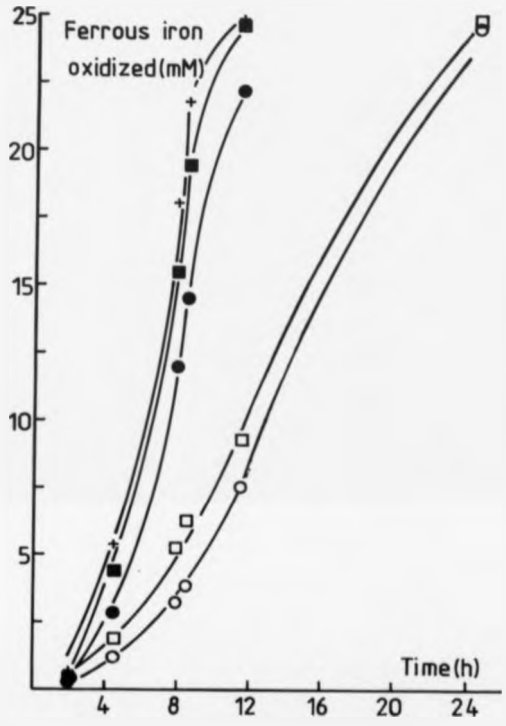


Fig 4.17
Comparison of growth curves as substrate oxidized against time during chemolithoheterotrophic growth of strains TH1 and TH3. Cultures were incubated at 48°C, in 100ml ES at pH1.8 with a 0.01%w/v yeast extract supplement in 250ml flasks shaken at 120rpm. Inocula were 4%v/v. Cultures were strain TH1 with 25mM ferrous iron, only(● - unaffected by 62.5mM sodium, not shown) and plus 125mM ferric iron(○); strain TH3 with 25mM ferrous iron, only(■) plus 62.5mM sodium(+) and plus 125mM ferric iron(□).

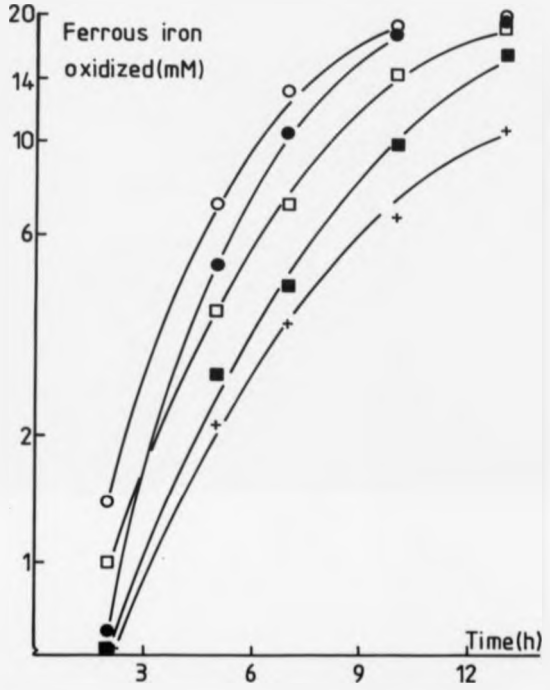


Fig 4.18
Effect of increasing ferric iron concentration on logarithmic rate plots of ferrous iron oxidation during batch culture of strain TH1. Incubation was at 48°C, in 100ml ES at pH1.8 with nutritional supplement of 0.01%w/v yeast extract. Flasks(250ml vol) were shaken at 120rpm. Inocula were 5%v/v. Growth was with 20.2mM ferrous iron, only(○), plus ferric iron at 12.1mM(●), 32 mM(□), 72.9mM (■) and 121.5mM(+). Sterile control (not shown) remained at a level of oxidation below the start of the scale used on the above graph.

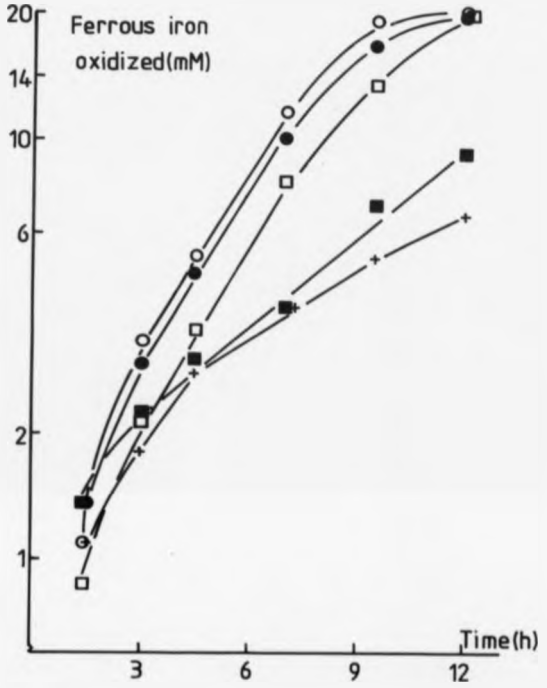


Fig 4.19
Effect of increasing ferric iron concentration on logarithmic rate plots of ferrous iron oxidation during batch culture of strain TH3. Incubation was at 48°C, in ES at pH1.8 with nutritional supplement of 0.01%w/v yeast extract. Inocula were 5%v/v. Flasks(250ml vol) were shaken at 120rpm. Growth was with 20.2mM ferrous iron, only (○), plus ferric iron at 12.1mM(●), 32mM(□), 72.9mM(■) and 121.5mM(+).

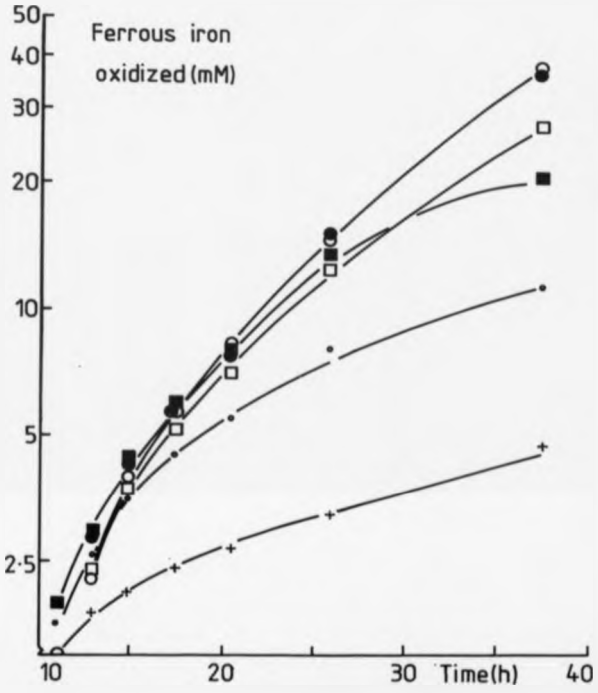


Fig 4.20
Logarithmic rate curves of iron oxidation during batch culture of strain ALV at increasing ferrous iron substrate concentration. Cultures in 100ml ES at pH1.7 were incubated at 45°C, in 250ml flasks shaken at 120rpm and aerated with 1%v/v CO_2 in air. Inocula were 6% v/v from an oxidized, 50mM ferrous iron culture. Initial substrate concentrations were 5mM(+), 10mM(•), 20mM(■), 30mM(□), 40mM(●) and 50mM(○) ferrous iron.

the length of the lag phase (fig 4.21). Oxidation in the presence of 72.9 mM ferric iron did not go to completion except under autotrophic conditions, slowly oxidizing over a 70 h period.

The little studied isolate, strain LM2 was severely limited by ferric iron during growth. Total inhibition of iron oxidation (with 20.2 mM ferrous iron) developed in the presence of 32 mM ferric iron, with no oxidation at all at the higher concentrations of 72.9 mM and 121.5 mM ferric iron (fig 4.22). In the absence of added ferric iron the oxidation rate slowed prior to 50% iron oxidized, occurring earlier in the growth curve on adding ferric iron.

4.3.3 Rate curves - Sulfolobus BC65

Sulfolobus BC65 from a pyrite grown stock culture was prepared through serial sub-culture on ferrous iron with 1 mM tetrathionate. During oxidation of 20.2 mM ferrous iron, a linear logarithmic rate plot estimated the doubling time at 5.3 h (fig 4.23). The presence of ferric iron increased the length of the lag phase and caused increasing decline in the maximum oxidation rate although the effect of 32 mM and 72.1 mM ferric iron were largely similar in this experiment. Growth with 121.5 mM ferric iron was more inhibited resulting in a more severe decline in oxidation rate and after relatively less ferrous iron had been oxidized. But, over a total of 50 h the culture reached 70% (14 mM) oxidized.

4.4 IRON OXIDATION IN CONTINUOUS CULTURE

During batch growth on ferrous iron the different strains exhibited patterns of oxidation in the presence of ferric iron that could in part be related to kinetic measurements with cell suspensions. Continuous iron oxidation systems were investigated in this study in two respects, firstly as a tool in further indicating the relevance of cell suspension data to growth and secondly to compare the capabilities of several organisms in re-generating ferric

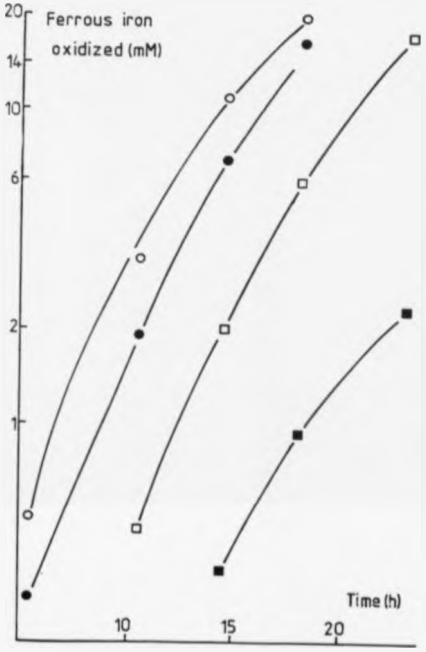


Fig 4.21
Effect of increasing ferric iron concentration on logarithmic rate plots of ferrous iron oxidation during batch culture of strain ALV. Incubation was at 45°C, in ES at pH1.8 supplemented with 0.01%w/v yeast extract in 250ml flasks shaken at 120rpm. Growth was with 20.2mM ferrous iron, only(○) and plus ferric iron: 12.1mM(●), 32mM(□) and 72.9mM(■). Oxidation with 121.5mM ferric iron was identical to sterile control(did not fit on above graph).

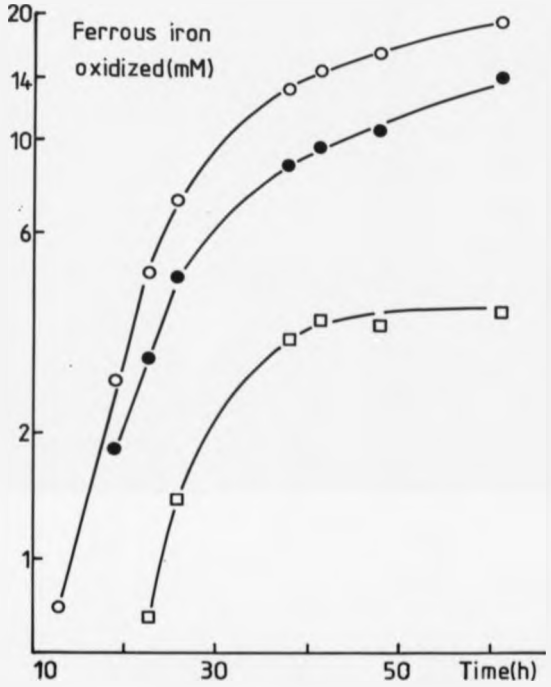


Fig 4.22
Logarithmic rate curves for iron oxidation by strain LM2 in batch culture and the effect of ferric iron. Medium was ES,pH1.7 supplemented with 1mM tetrathionate and aerated with 1%v/v CO_2 in air. Incubated at 48°C flasks(250ml vol) were shaken at 120rpm and inoculated 5%v/v. Iron substrate concentration was 20.2mM ferrous iron(○) plus 12.1mM(●) and 32mM(□) ferric iron. Oxidation in the presence of either 72.9mM or 121.5mM ferric iron was identical to the sterile control (not shown).

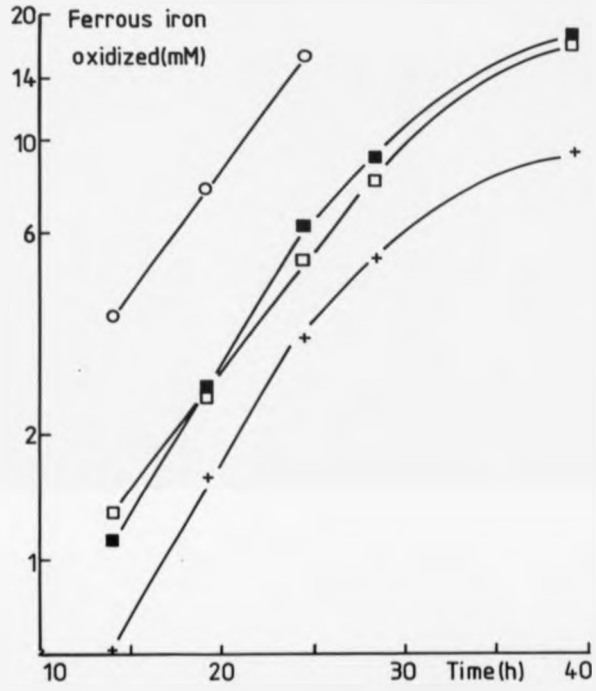


Fig 4.23
Logarithmic rate curves showing the effect of ferric iron on ferrous iron oxidation by Sulfolobus BC65 in batch culture. Medium, ES at pH1.7 was supplemented with 1mM tetrathionate and aerated with 1%v/v CO_2 in air. Inocula, 2%v/v were taken from an iron grown culture in it's 5th sub-culture on ferrous iron from a pyrite grown stock culture. Flasks(250ml vol) were shaken at 120rpm at 68°C. Oxidation was measured for 20.2 mM ferrous iron. alone(○) plus 32mM (□), 72.1mM(■) and 121.5 mM(+) ferric iron. Sterile control was below the scale of the graph.

iron oxidant. For both points a major aim was to consequently develop data for systems not operated exclusively with T.ferrooxidans.

4.4.1 Biomass retention of L.ferrooxidans

4.4.1.1 Observations on auto-aggregation in L.ferrooxidans

Both L.ferrooxidans and the Leptospirillum-like bacteria formed cell aggregates in ferrous iron grown batch cultures (Harrison and Norris,1985;1.8.1). Though the aggregates appeared to consist of slime-embedded cells, neither the slime layer nor the physical conditions of it's production and therefore of aggregation were characterized. The ability of aggregates to oxidize iron and the implications for ferric iron production were tested.

During batch culture, iron oxidation continued in the presence of aggregation, which increased over time. Aggregates resuspended in fresh media both oxidized the iron and increased in bulk. No evidence of any ferric iron precipitation associated with the aggregates was observed. Individual aggregates of up to 1-3 mm by 0.5-1 mm size were crushed on glass slides. Microscopically, a dense concentration of bacterial cells was observed. These cells as isolated aggregates, displayed oxygen uptake when placed with ferrous iron in the oxygen electrode (at 30°C). This confirmed the retention of ferrous iron oxidizing activity. The general form of the aggregates appeared to be strain specific.

With shake flask cultures aggregation of L.ferrooxidans was consistently induced at either 15°C or 25°C. These sub-optimal growth temperatures consequently increased the doubling time and rate of iron oxidation. The use of normal agitation (shaking at 120 rpm) and an incubation temperature of 30°C largely prevented aggregation. Under these conditions aggregation did occur in 1 l medium volumes shaken in 2 l flasks. In these flasks aggregates were poorly distributed and were probably evidence of areas of poor mixing in the base of the flasks. The appearance of aggregates under these conditions was not

consistent and generally formed smaller sized clumps than those in low agitation cultures and at 25°C.

Using low agitation during early growth phase aggregates were produced in batch fermenter vessels at 25°C. On oxidising the available ferrous iron this culture produced pin-head size particles in the vessel showing that batching up of macroscopic biomass in culture vessels prior to switching to continuous culture was possible.

4.4.1.2 Process design

The physical process problems of maintaining aggregations in a continuous oxidation system had to be considered. The experimental system was continually adapted as a necessary part of investigating the operation of a high biomass retention system. The problems encountered were partly caused by the small scale nature of the experimental system. The continuous system was developed using a 1 l glass vessel of 700 ml working volume with drainage from a weir type overflow (operational details 2.2.3). Batch operation of the vessel provided macroscopic biomass prior to switching to continuous flow. The effluent, including aggregates, was collected in a separating funnel before running into the final waste pot but the inflow of effluent maintained sufficient currents to prevent settling. After considering the process complications of settling, centrifugation and filtration prior to biomass recycling, attention was switched to restricted out-flow of the aggregated biomass. A sintered glass sparger was fitted into the vessel via the lid through which the effluent was blown out by the positive pressure of the incoming air(by sealing the gas exhaust). The volume level was maintained and the biomass was concentrated although the 'filter' was not 100% efficient. The attachment of biomass to and loss via the sparger increased with increasing flow rate as the liquid level made a more complete solid/liquid interface prior to being blown out. Attached biomass, eventually blocked the sparger and therefore the gas exhaust. Back pressure caused oxygen limitation and a reduction in ferrous iron oxidation. Breakdowns in physical process required

recovery periods for the system to recover (discussed in 4.4.1.3). The effluent was subsequently pumped out via the sparger and an independent gas exhaust used. The percentage of total biomass present as aggregates at any one time could not be determined. Not finally resolved with regards to problems of process the finalized "system" did provide data for the manipulation of this microbiological phenomenon and the effect on ferric iron production.

4.4.1.3 Experimental data

Figure 4.24 gives an overview of the operation of the system both as ferrous iron oxidized (mM) and as flow rate ($ml h^{-1}$) against time. This relationship was altered by the physical process variations and upsets described in 4.4.1.2 (vessel design and operation,2.2.3). Substrate concentration was changed from 50 mM to 100 mM when oxidation efficiency remained at greater than 90% ferrous iron oxidized during prolonged flow rate increases. This aided the aim of increasing ferric iron production. Change in operational data also coincided with pH adjustment of the medium in order to maintain a constant pH in the vessel below pH 1.6 to prevent iron precipitation. As pH control was not automatic adjustment of the reservoir was required. Increase in the acidity of the medium inflow caused transient drops in ferrous iron oxidation. Recovery periods from any interruption were effectively short batch culture phases of a few hours duration. Ferrous iron oxidation was allowed to return to previously high levels prior to interruption before restarting continuous medium flow. This was done in order to concentrate on the influence of retained biomass within the vessel. The ability to resume the same flow rate after % oxidation levels had increased demonstrated the rapid recovery of the system (due to the captive biomass). During satisfactory operation flow rate was increased with no effect on the concentration of residual ferrous iron in solution. Both these values combined revealed a significant change in the total concentration of ferric iron produced. The productivity of ferric iron as mg per h was compared with protein concentration (µg protein per

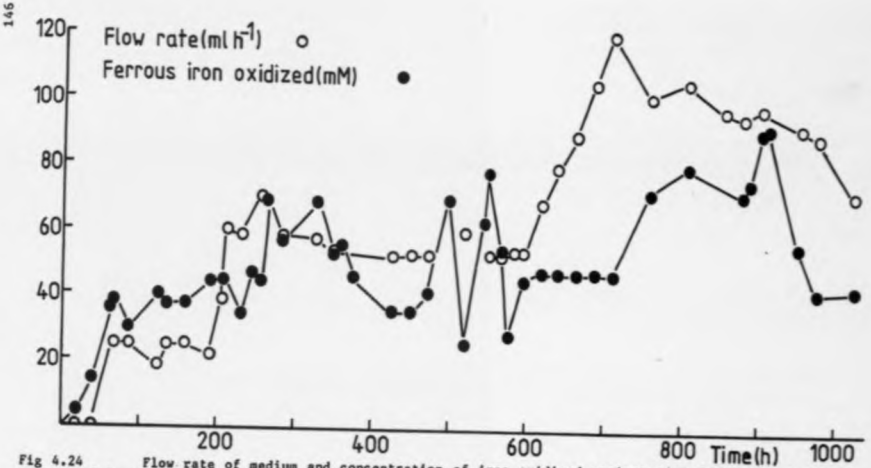


Fig 4.24 Flow rate of medium and concentration of iron oxidized against time during continuous culture of Leptospirillum ferrooxidans, with biomass retention, on ferrous iron. Medium, 700ml ES, was kept below pH1.6 at 25°C. Inflow ferrous iron substrate concentration was either 50mM or 100mM. Vessel, magnetically stirred was sparged aseptically with 5%v/v CO_2 in air, 30ml per min. Break at 500h indicates dis-continuous operation.

ml in the reaction vessel - protein sample and measurement 2.2.4) against time (fig 4.25).

In the first 250 h of operation, including the initial batch up phase the aggregates increased in size from their initial tiny form. Increased agitation appeared to prevent further increase in the size of aggregates but did not decrease the size or degree of aggregation. The protein concentration remained fairly constant at approximately 7.5 µg per ml before increasing at about 200 h matched by an increase in ferric iron productivity (fig 4.25). Matching trends of rise and fall and therefore causation for many of them are reflected in fig 4.24. The flow at around 200 h was increased as the degree of iron oxidation remained beyond 80%. Comparing figs 4.24 and 4.25, the productivity of ferric iron also increased as the initial substrate concentration was raised from 50 mM to 100 mM at around 250 h. Despite this, protein concentration which had significantly increased from 27 µg per ml to 39 µg per ml was reduced to 22 µg per ml, slowly recovering over the next 100 h (fig 4.25). This recovery was transient with a further loss of protein at about 350 h initiated by an adjustment in pH and a reduction in ferrous iron oxidation (fig 4.24). This suggested that protein concentration in the vessel was dependent on iron oxidation. If the oxidation was prevented (inhibited) the energy being generated would not be sufficient to maintain the population. This implied that protein concentration was not an inactive artefact of biomass retention but an indicator of active oxidation potential.

This protein loss was followed by air limitation of the system. Continual process problems some 400 h into the experiment resulted in a restart of the system, with pumped out overflow and 50 mM ferrous iron, using the viable biomass left in the vessel (at 550 h, fig 4.24). This resulted in a further 300 h of uneventful operation (from 600 h to 900 h) with increasing productivity by which time flow rate approached limiting values for this system, despite being reduced in specific value on the re-introduction of 100 mM ferrous iron substrate concentration at 720 h (fig 4.24). The glass sinter on

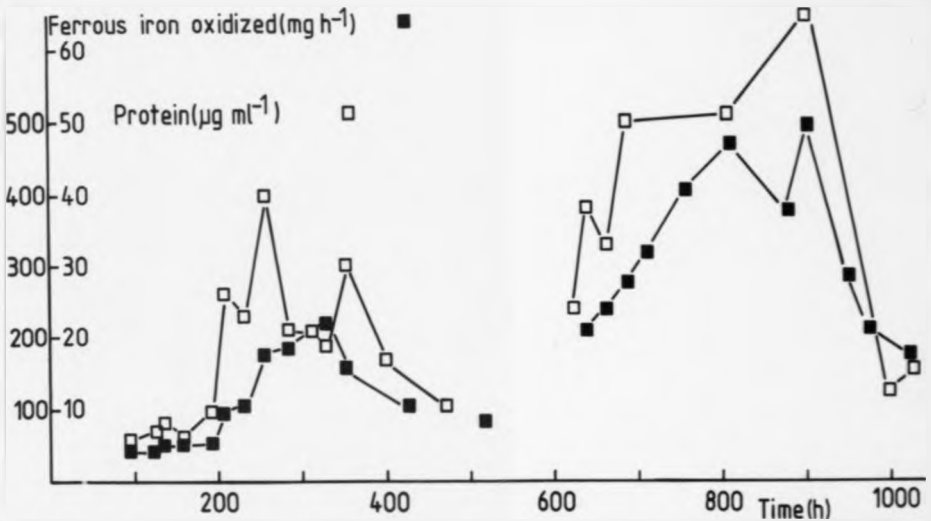


Fig 4.25 Comparison of productivity (as iron oxidation rate, left hand scale) and biomass concentration (as protein per unit volume) over time during continuous culture of L.ferrooxidans, with biomass retention, on ferrous iron. Medium, 700ml ES_1 was kept below pH1.6 at 25°C, and aerated 30ml per min with 5%v/v CO_2 in air. Substrate concentration was either 50mM or 100mM ferrous iron. Discontinuous operation at 500h not recorded. (Data complementary to fig 4.24).

the outflow became continually coated with biomass both on the glass and through the pores and had to be replaced daily after washing in hot acid. (By this time sterile operation was consequently completely broken, despite precautions, but contamination was not thought likely to become a problem given the concentration of the original monoculture biomass). This coating threatened to block the sinter which did occur at 340 h causing a reduction in productivity (fig 4.25). Eventually, the proportion of biomass on the sinter increased, ferrous iron oxidation dropped and the system completely failed with the consequent lack of recovery apparent from the massive proportional loss of protein in suspension from 63 µg per ml to 12 µg per ml in 100 h (fig 4.25).

As the limits of productivity were being investigated kinetic data for steady states was not measured. However, from 600 h to 720 h greater than 90% oxidation was maintained at increasing flow rate reaching a maximum of 119 ml per h prior to increase of inflow substrate concentration. This was equivalent to a dilution of 0.17 h^{-1} the final productivity of the system peaking at 490 mg ferric iron per h (fig 4.25). In fig 4.26 productivity is expressed as a relationship rather than as a comparison with protein concentration. This illustrated proportionality of productivity relative to protein concentration retained in the vessel.

4.4.2 Biomass retention of T.ferrooxidans - Bacfox-type reactor and effect of pH

Continuous iron oxidation of T.ferrooxidans was studied in conditions that allowed the precipitation of ferric iron, based on the operation of the Bacfox process (1.6.3). The influence of available surface area and pH were studied (vessel design and operation,2.2.3). The medium, kept in sterile glass reservoirs was enriched salts containing 5 g per litre ferrous iron (as 24.9 g per litre (89.6 mM) ferrous sulphate). Two vessels were operated within the pH limits of ferric iron precipitation, one with internal glass tubes and one without. A third vessel complete with internal tubes was operated without allowing ferric iron

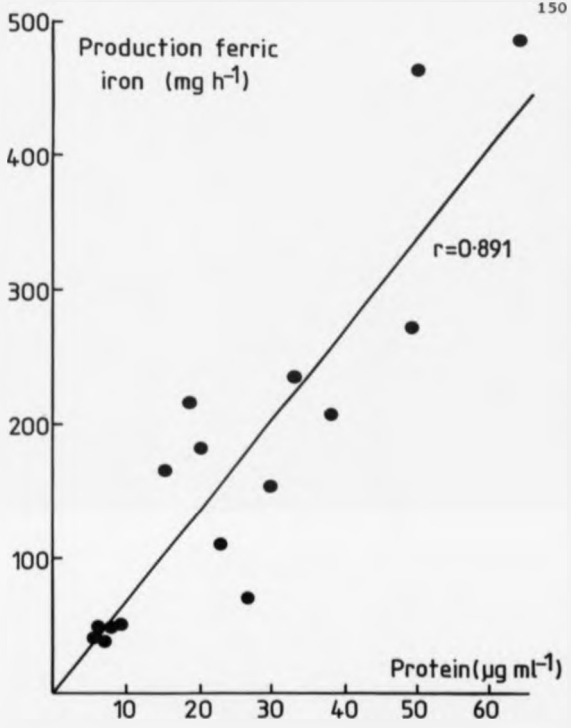


Fig 4.26
Relationship of iron oxidation (as ferric iron production in unit time) against protein concentration (per ml of culture volume) during continuous culture on ferrous iron of L.ferrooxidans. Magnetically stirred 1 l glass vessel kept below pH1.6, at 25°C for 700ml vol of ES medium. Aerated with 30ml per min 5%v/v CO_2 in air in-flow substrate concentration was changed between 50mM and 100mM ferrous iron. Correlation coefficient, r shown for the plotted linear relationship.

precipitation. This was firstly a control on the influence of precipitation (the acidity) on iron oxidation, and secondly, an attempt to form a true biofilm of T.ferrooxidans in one not associated with iron precipitates. The vessels were not operated as chemostats, iron oxidation never being consistently high enough to allow inflowing ferrous iron concentration to be at the level of a limiting substrate. The dilution rate was an important parameter given the small scale of the experimental apparatus. This caused difficulty in providing slow enough pumping rates, initially, prior to the establishment of a stable iron oxidizing culture. Daily flow rates and measurement of percentage iron oxidation were recorded for all three vessels. The operation was under non sterile conditions.

(At high flow rate the value of specific flow rate measured was found to be inaccurate based on the turnover of total medium volume from the reservoirs. Flow data and reservoir changes before and after the highest flow rate values agreed accurately . With flow data and reservoir changes agreeing, the data for the first 800 h of operation is presented, the data during and after the discovered inaccuracy is not, although observations are recorded on subsequent apparent operation of the vessels).

On average, for both vessels supporting iron precipitation, the medium pH was pH 1.65 rising during oxidation to pH 1.98 (higher inflow pH values resulted in total precipitation within the vessels). To prevent precipitation the third 'acid' vessel had an input of pH 1.41 which increased to pH 1.56. In all three vessels the oxidized medium was clear and red/brown. All the glass surfaces, in contact with the culture medium (except the base) at pH 1.98 became clouded in the first few days of operation with an off-white precipitate (it's chemical/biological composition was not investigated). A rapid build up of red brown precipitates was subsequently supported by the glass, throughout the experiment, progression visually slowing over time but not stopping.

Without added surface area precipitation covered all the

glass surface. Over 800 h of operation this allowed an increasing flow rate to result in an overall increase in productivity of ferric iron (fig 4.27). During this time although averaging at above 70% oxidized the degree of oxidation maintained a downward trend. The level of oxidation was unstable both due to the inconsistent nature of operational parameters ie increasing flow rate, and because of the instability of the precipitate layer. The instability resulted in changeable plots in flow data with percentage iron oxidation peaking due to a reduction in flow rate, then falling on increasing rate but when rate was stabilized percentage oxidation both recovered and stabilized over further flow increases (fig 4.27, 375 h). The maximum ferric iron production achieved was 352 mg per h at a dilution rate (D) of 0.23 h^{-1}. The production level was erratic during the experiment.(After the 800h period efficiency of the vessel decreased. Simultaneously the precipitate layer became unstable leaving large areas of bare glass. This loss of support for the cells appeared to reduce the capacity of the vessel for oxidation despite decreases in flow rate.)

The flow rate data was more erratic for the vessel with tubes (pH 2). Percentage iron oxidized was not constant causing fluctuations in iron production (fig 4.28). It did return consistently to 80% oxidized as the flow rate was increased. This suggested that fluctuations were caused by the recovery periods being too short between changes in flow rate. The maximum calculated production of ferric iron was 602 mg per h at an equivalent D of 0.43 h^{-1}. Beyond the 800 h period increases in dilution rate caused the oxidation level to decrease. Stability of the vessel failed with reduced recovery on lowering the flow rate. This instability indicated that stable production of ferric iron was probably below the maximum level observed. That is, maximum production in a stable system would probably have been of the order of 500 mg ferric iron per h.

Fig 4.27
Flow rate of medium and % iron oxidation (left hand ordinate scale) against time during continuous culture on ferrous iron of T.ferrooxidans. Combined, the production of ferric iron is given on the right hand ordinate scale. Vessel was magnetically stirred, under non-sterile conditions at 30°C for a 390ml working volume of ES at pH2. Inflow ferrous iron concentration was $5gl^{-1}$. Operation was restarted after initial flow rate was started too high. Medium was aerated at 40ml per min.

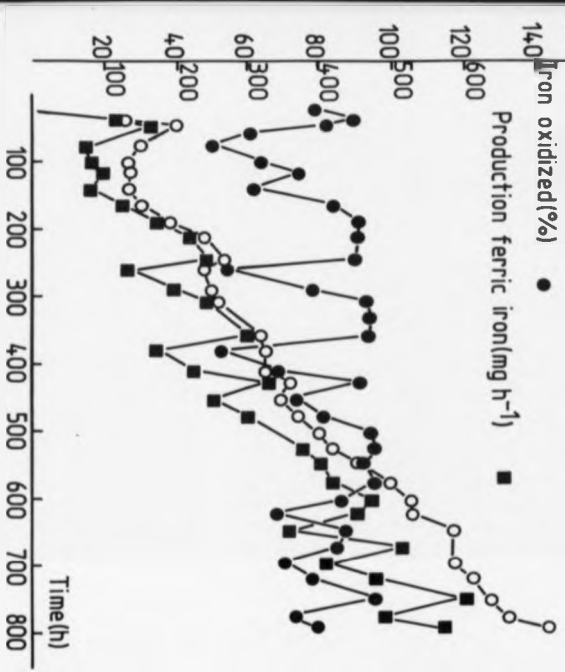


Fig 4.28
Flow rate of medium and % iron oxidation (left hand ordinate scale) against time during continuous culture on ferrous iron of T.ferrooxidans in a Bacfox model. The culture in 290ml ES, maintained during oxidation at pH2, was magnetically stirred and sparged 30ml per min with air during incubation at 30°C, under non-sterile conditions. Substrate inflow was $5gl^{-1}$ ferrous iron. Continuous production of ferric iron (right-hand scale) was calculated for all sample points. The vessel was fitted internally with glass tubes supported above the stirrer on a perforated perspex base. This increased internal surface area of vessel used for fig 4.27 by greater than x4.

The third oxidation vessel, with tubes added but an average pH during oxidation of pH 1.56, was the most stable in the attempt to achieve an average of 70% iron oxidation (fig 4.29). Flow rate, and therefore ferric iron production, was low, relative to the other two vessels. The gentle increase in flow rate may have indicated a slow adaptation to the acidic conditions. During this period the glass remained clear with no evidence of precipitation. In 800 h of operation this gave a maximum ferric iron production of 131 mg per h at a 0.13 h^{-1} dilution rate. (During subsequent operation one change of reservoir allowed a transient pH rise in the vessel to above pH 1.8 at which time the glass clouded with an off white precipitate prior to pH correction. This precipitate remained in the vessel at below pH 1.6 but did not appear to increase and no amorphous masses of red-brown precipitates appeared. However productivity increased by 50% due to flow rate increases both of which fell in the 100 h period after return to acid pH, but not to as low a level before interruption in pH. By this time strands of material of apparent microbial biomass were formed as streamers on portions of the glass - these were few in number and were not investigated given the relatively poor performance of this vessel overall).

4.4.3 Comparison of continuous iron oxidation by T.ferrooxidans and strain ALV

Strain ALV was the only organism (in this study) capable of complete iron oxidation in the same nutritional conditions as T.ferrooxidans (3.2.4). Complementary to cell suspension studies (4.2.4) strain ALV was compared in a study of continuous iron oxidation with T.ferrooxidans. to measure the kinetic performance of the moderate thermophile. The vessels used were operated aseptically. The air supply was enriched to 1% v/v carbon dioxide in air (because of the requirement of strain ALV). Fresh medium was passed via glass or flexible pvc tubing due to the toxicity of silicone rubber to strain ALV (fig 2.3). The overflow was blown out at around 500 ml volume,

Fig 4.29
Flow rate of medium and % iron oxidation (left hand ordinate scale) against time during continuous culture on ferrous iron by T.ferrooxidans in a Bacfox model operated at acid pH. Production of ferric iron measured on the right-hand vertical scale. Working volume of 290ml ES was incubated at 30°C and magnetically stirred. With initial substrate concentration of 5gl^{-1} ferrous iron, medium was kept below pH1.6 during oxidation. Vessel was aerated under non-sterile conditions at 30ml per min air. Internal surface area was increased by glass tubes supported above the stirrer on a perforated perspex base.

without incident (2.2.3).

Strain ALV was grown with 50mM ferrous iron and a pH during oxidation of between pH 1.55 and pH 1.60. Complete oxidation was recorded during batch growth but was not repeated in just under 400 h of continuous operation. On switching to continuous flow, even at a D of 0.03 h^{-1}, percentage oxidation dropped to 70%. Increases in D dropped percentage iron oxidized to less than 30%. This decline was halted and reversed by decreasing flow rate. At 30% iron oxidized continuous flow was stopped and during subsequent batch growth oxidation of ferrous iron was completed. Growth then appeared possible in continuous operation but at very limited levels. Accepting the limitations of strain ALV, observed above, a more complete investigation of it's performance during continuous iron oxidation was attempted under the same conditions at 45°C. The performance of this vessel was plotted as flow rate and percentage iron oxidized over the time course of the experiment, in comparison with T.ferrooxidans (fig 4.30). The oxidation capacity of the vessel could not be maintained at above 50% ferrous iron oxidized. The iron oxidation level was sensitive to slight alterations in flow rate and was then slow to stabilize. This made the identification of steady states difficult. The effect of flow rate and therefore of dilution rate on apparent oxidative capacity was, however , clear. Increased flow rate resulted in a drop in iron oxidation apparently stabilizing between 80 h and 200 h of operation (fig 4.30). This slow stabilization (approximating to the first steady state) was followed by the oxidation level falling below 20% on further increasing the flow rate. This level was sustained approximating to a second steady state up to 360 h. Beyond this point a slight reduction in flow rate was followed by a continuous but slow increase in oxidation from 10% to 35% oxidized in almost 200 h of operation by which time it appeared to level off. Another small flow rate drop (approximately 2 ml per h) sustained this slow increase (650 h, fig 4.30). Within a few per cent of ferrous iron oxidized, this period between 660 h and 760 h could be

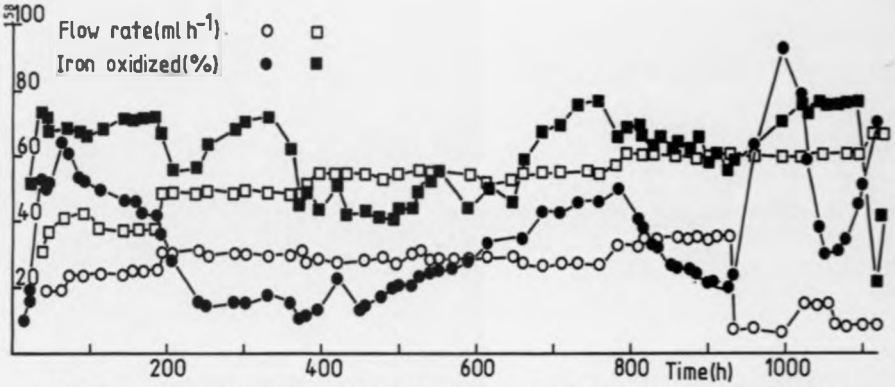


Fig 4.30 Comparison of medium flow rate and % iron oxidized against time during continuous culture on ferrous iron of T.ferrooxidans(■,□) at 30°C and strain ALV(●,○) at 45°C. Both vessels contained 500ml ES with 50mM ferrous iron substrate, kept below pH1.6. Sterile air with 1%v/v CO_2 was used at 50ml per min. Vessels were magnetically stirred.

called the third steady state. Another small increase in flow rate caused a definite drop in the percentage of iron oxidized. To finish the experiment, as a check that the medium was limiting only as the rate of input, flow rate was reduced below 10 ml per h at which point oxidation increased to greater than 90% oxidized (fig 4.30). This level of oxidation was manipulated by altering the flow rate. The oxidative capacity of strain ALV was therefore low during continuous iron oxidation, the greatest productivity being 44 mg ferric iron per h. Steady states were, as mentioned above, difficult to identify but using the three mentioned and the flow rates at which the oxidation level latterly reached it's two highest levels of 93% and 81% oxidized the relevant dilution rates were calculated and plotted relative to the measured iron oxidation level. This showed a steady decline in oxidation with increasing dilution rate (fig 4.31).

In comparison T.ferrooxidans was capable of a sustained level of oxidation (70%) at increasing dilution rate to values beyond the apparent ability of strain ALV (fig 4.30). Initially the oxidation level stabilized at around 67% and increased to 72% following a reduction in flow rate up to 180 h total operation. At a D of 0.097 h^{-1} (at 400h) the oxidation level dipped but recovered over the extended operation at this flow rate. However, in conjunction with erratic levels of percentage iron oxidized the level recovered with no significant flow rate changes recorded. This resulted in two very different levels of oxidation at the same dilution rate (figs 4.30, 4.31). The second value was an average from a still increasing level of percentage oxidation. The pattern was repeated by flow rate adjustment to 60 ml per h with oxidation slowly decreasing, increasing and then levelling off. At each stage this suggested a recovery or adaptation period but without any apparent cause other than due to flow rate. This was similar to the Bacfox vessel of 4.4.2 except that the recovery of oxidation after flow rate adjustment was slower. The highest recorded ferric iron production was 127.2 mg ferric iron per h at a dilution rate of 0.12 h^{-1}.

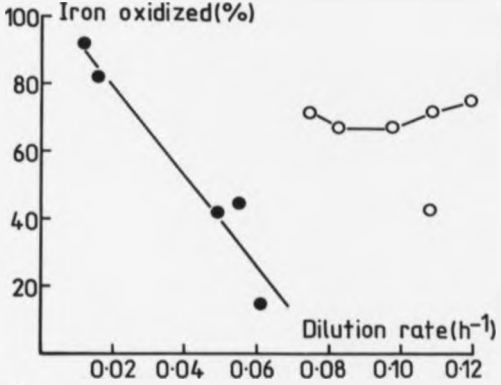


Fig 4.31
Comparison of % iron oxidized against dilution rate during continuous culture on ferrous iron for strain ALV(●) and T.ferrooxidans(○). Growth was measured in water-jacketed vessels at 45°C and 30°C respectively, magnetically stirred and aerated with sterile air containing 1%v/v CO_2 at 50ml per min. Medium was 500ml ES held below pH1.6, with 50mM ferrous iron substrate in the inflow. Plots of % iron oxidized are for samples taken during what approximated to steady states (minimum of three volume changeovers) or during periods of greatly reduced oxidation (strain ALV), indicating apparent wash-out. The outside plot for T.ferrooxidans indicates an alternative level of oxidation for the same dilution rate measured at different points in the experiment.

4.4.4 Competition during continuous iron oxidation between T.ferrooxidans and L.ferrooxidans

Competition between different acidophiles was attempted in order to demonstrate the relevance of cell suspension apparent K_m data to growing cultures. To investigate the competitive advantage of apparent K_m required two species (for simplicity) having similar physiology but divergent affinity for ferrous iron substrate. Sulfolobus BC65 was not considered being the only thermophile. Choices existed from the moderate thermophiles. Strain ALV with an apparent K_m of 2.96 mM was nearly three fold different from strain TH1 of apparent K_m 1.03 mM. However, the response of strain ALV to ferric iron, during oxidation by suspension (4.2.4), during batch culture (4.3.2) and in continuous culture (4.4.3) suggested that at high ferric iron concentration it might be permanently inhibited and struggle to grow irrespective of the influence of competition. Strain TH3 had an essential growth requirement for yeast extract which being facultatively utilised by strain TH1 presented the possibility of limitation of and competition for the yeast extract irrespective of response to ferrous iron levels. On this basis the mesophiles were chosen with measured apparent K_m values of 0.25 mM and 1.34 mM for L.ferrooxidans and T.ferrooxidans respectively.

Experimental conditions had to provide data reflecting cell response to environment, based on inherent kinetic values, including cell washout at dilution rates greater than growth rate. Selective pressures that might alter this were prevented. The pH was kept below pH 1.6 preventing precipitation and biomass retention (cf 4.4.2) and to prevent auto-aggregation of L.ferrooxidans efficient mixing and near optimal growth temperatures were used (cf 4.4.1.1). With optimal temperatures of L.ferrooxidans and T.ferrooxidans of 35°C and 33°C respectively (temperature comparison,appendix 3) a compromise incubation temperature of 32°C was used.

Two cultures of each mesophile were grown in four separate glass vessels of 400-550ml operating volume. Magnetically

stirred each vessel had a blow out overflow. This was found to pull the medium (enriched salts, 50 mM ferrous iron) out of the pipettes placed in the feed line to measure flow rate. On discovering the inaccuracy the volume of overflow was measured over time to indicate flow rate. This, however, resulted in the loss of the first several hundred hours of continuous flow data.

The vessels were stabilized at low flow rate at 90% to 95% oxidized for both T.ferrooxidans cultures and 95% to 100% for both L.ferrooxidans cultures. At this point one of each vessel was equally mixed relative to volume and maintained at the same flow rate conditions leaving two monocultures and two mixed cultures. At approaching steady states of flow rate and percentage iron oxidized the separate vessels were sampled. This was to indicate both protein content and relative cell numbers and compare the effect of flow rate on the performance of individual monocultures and mixed cultures.

The measurement of relative species number in the mixed suspensions was difficult. Morphologically distinct the two species of mesophile were difficult to distinguish proportionately under a microscope particularly under dense growth conditions and/or where one organism was dominant. Without a specific stain or immunological test for each organism, investigation was made of distinguishing between the different physiologies by the proportionate response of iron oxidizing activity to specific inhibitors. At the same concentrations of copper and nitrate for each organism T.ferrooxidans was more sensitive to nitrate and L.ferrooxidans was more sensitive to copper (Eccleston et al.1985). This presented the possibility of using differential inhibition to estimate the proportions of each organism in a mixed culture. For a mixed culture the two cell populations were expected to change with environmental conditions, giving two mathematical variables X and Y, the concentrations (as %) of L.ferrooxidans and T.ferrooxidans respectively. In any culture, activity would be due to X + Y and would be proportionately reduced by the specific activity of an inhibitor. By example - copper inhibited oxidation rate

= aX + bY where a and b were the percentage of inhibition caused by the same concentration of copper for pure cultures. On constructing a similar equation for nitrate inhibition the unknowns X and Y could be derived by the method of simultaneous equations. (This step is worked out in full in appendix 4). Samples of 100 ml volume were prepared as cell suspensions (2.3.1) to an optical density of 0.47 at 540 nm. The extrapolation of proportional cell numbers from differential inhibition included several assumptions. Firstly, any physiological interaction of either copper or nitrate was not tested. The assumption was that selective binding or adsorption did not occur by one species, reducing the inhibition to the other. Secondly it was assumed that neither mesophile would interact as a result of (organic) material leached from the cells into the medium. (No effect was seen for batch growth curves in spent filtered culture media supplemented with fresh iron.) Thirdly the small number of activity measurements made from the small cell yield from a 100 ml sample led to variation in the arithmetic of the simultaneous equations (appendix 4). It was assumed that the ratio of percentage activities within a cell mix accurately corresponded to cell ratios despite the failure of total percentage activity to equal 100%.

The flow rate and the percentage iron oxidized were measured against time and graphically plotted for L.ferrooxidans (fig 4.32). The vessel was sampled to give a (first) base point after the first period of inaccurate flow measurement. The percentage iron oxidized steadily decreased to 55% over the next 80 h as flow rate increased (650 h,fig 4.32). With a slight flow reduction ferrous iron became limiting and a steady state measurement (minimum of three volume changes) was taken. Oxidation decreased following a second sharp flow rate increase but stabilized (this was taken as the third measurement point though no protein sample was taken). At around 840 h clumps of biomass began to be observed in the medium and increased as percentage iron oxidized decreased over a 100 h period. Oxidation decreased to 30% before flow rate reduction allowed recovery to a level of ferrous iron

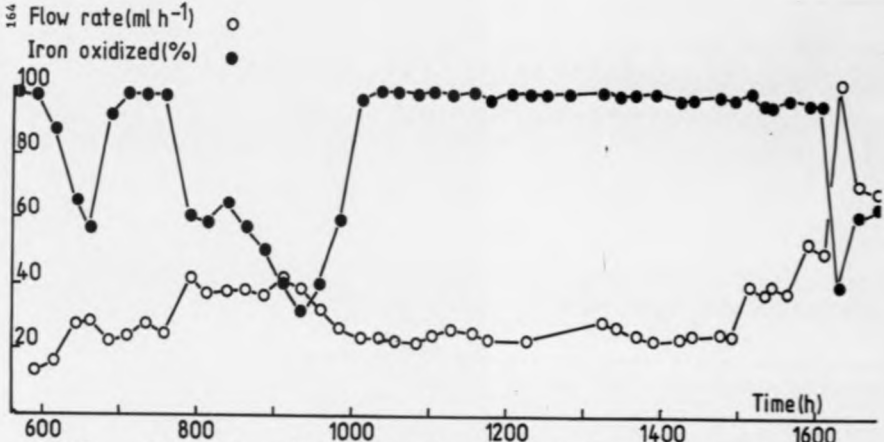
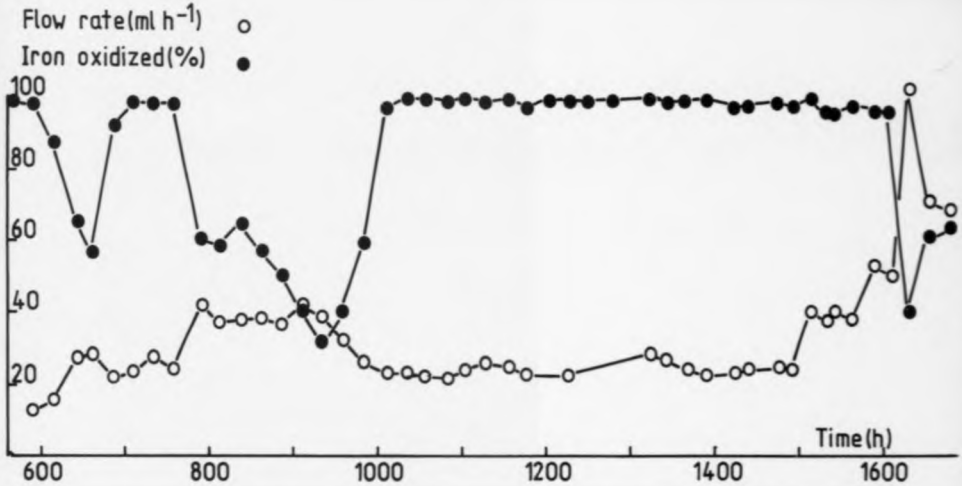


Fig 4.32 Percentage iron oxidized and medium flow rate against time during continuous culture on ferrous iron of L.ferrooxidans. Enriched salts medium (410ml) with 50mM ferrous iron substrate was kept below pH1.6, at 32°C. Magnetically stirred vessel was aerated at 50ml per min. The initial part of the experiment is not plotted due to discovered inaccuracy in measured flow rate.



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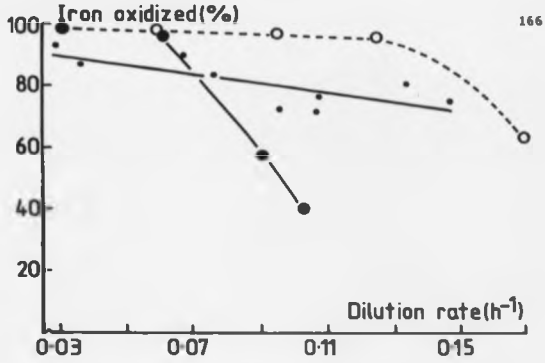
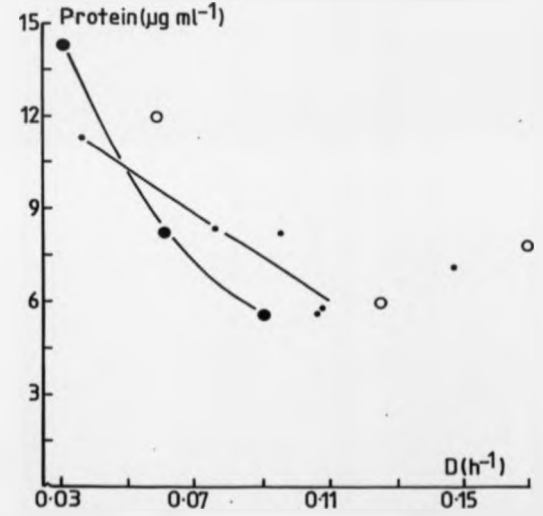
limitation. Near the end of the oxidation decline the vessel was sampled. When plotted as percentage iron oxidized against dilution rate (fig 4.33) the implication was that the L.ferrooxidans vessel was washing out at D greater than 0.1 h^{-1}. This was supported by the continued decrease in oxidation before the flow rate was lowered and by the decline in protein content at the same dilution rates (fig 4.34). After 1000 h of total operation the visible concentration of biomass stabilized quickly and the vessel was maintained for several hundred hours under ferrous iron limitation, to provide pure culture samples for the differential inhibition test.

At 1180 h a steady state sample was taken (fig 4.33). Taken under similar flow conditions as sample 2, the corresponding protein content was significantly higher (fig 4.34) due to the biomass concentration. This raised several points. Firstly, why under optimum conditions had the organism aggregated? Secondly, why with an unrestricted overflow was biomass concentrating in the vessel? Thirdly, why if during apparent washout as above, aggregation was beginning to occur and increase, was washout in fact taking place? The answer to the first point was not obviously apparent. The aggregates increased in size and appeared to be permanent once they had occurred. The clump length approached the bore size of the open outflow tube (less than 5 mm). The liquid being blown out formed a permanent meniscus at the overflow . Surface tension and electrostatic forces may have prevented passage of biomass except at higher flow rates (greater pressure). For the third point a decreasing concentration of total protein may have been increasingly aggregating (relevant to the first point in that aggregation may not be caused by specific factors such as incubation temperature but by stress of environmental factors, here being high flow rate/concentration of substrate). As the experiment neared completion an attempt was made to wash out the cells. Percentage iron oxidation was little changed by a flow rate increase from 25 to 39 ml per h and then to an average of 51 ml per h. The vessel was sampled followed by a sharp rise in

Fig 4.33
Comparison of the relationship of % iron oxidized against dilution rate during continuous culture on ferrous iron of L.ferrooxidans and T.ferrooxidans. Medium for each culture was enriched salts, with 50mM ferrous iron substrate, kept below pH1.6 during oxidation, incubated at 32°C. Vessels were aerated at 50ml per min. Plots are for L.ferrooxidans, before(●) and after(○) the appearance of cell aggregates in the vessel, and for T.ferrooxidans(▪). The hatched line indicates an alternative relationship of dilution rate to oxidative capacity for L.ferrooxidans in the presence of aggregates. Culture volumes were 410ml(L.ferrooxidans) and 510ml(T.ferrooxidans). (Flow rate data against time presented in figs 4.32, 4.35).

Fig 4.34
Relationship of protein concentration in suspension to dilution rate during continuous culture on ferrous iron of L.ferrooxidans, before(●) and during(○) the formation of cell aggregates, and for T.ferrooxidans(▪). Culture was in magnetically stirred vessels at 32°C, in enriched salts medium with 50mM ferrous iron substrate kept below pH1.6 during oxidation. Vessels were aerated at 50ml per min. Plotted lines are arbitrary interpretations of the data. (Plots of protein concentration are complementary to the plots of % iron oxidation in fig 4.33).


Iron oxidized(%)
100
80
60
40
20
Dilution rate(h^{-1})
0·03
0·07
0·11
0·15


Protein(μg ml^{-1})
15
12
9
6
3
D(h^{-1})
0·03
0·07
0·11
0·15

dilution rate to 0.25 h^{-1}. Oxidation fell to 40% (fig 4.32). As the rate was approaching limitation it was reduced in order to estimate the dilution rate at which 95% or greater oxidation could not be maintained. The oxidation decline was halted and the final sample taken. In this vessel the maximum iron production was 140 mg ferric iron per h at a D of 0.125 h^{-1}.

When iron oxidation was plotted against dilution rate in fig 4.33 for the period with cell aggregates the plots indicated that resistance to washout had increased. The evidence of protein concentration in the vessel during aggregation was not clear (fig 4.34) other than showing a maintenance of protein (cells) within the system.

Changes in percentage iron oxidized in the T.ferrooxidans monoculture closely followed changes in flow rate (fig 4.35). More than 10% (approximately 5 mM) ferrous iron was unoxidized in the vessel at any given time. The performance of the vessel as percentage oxidation against dilution rate was plotted in comparison to L.ferrooxidans (fig 4.33). The level of oxidation declined with increased dilution rate but was sustained beyond the limits for L.ferrooxidans prior to aggregation (fig 4.33). The corresponding protein concentration also declined (fig 4.34). The apparent recovery of protein concentration at high dilution rate (at the end of the experiment) coincided with the appearance of large masses of apparent biomass in the vessel. (Identity of this material was not confirmed experimentally). This material appeared as loose flocculations. This extended the dilution rate at which T.ferrooxidans was maintained but did not increase ferric iron production beyond the maximum observed of 180 mg ferric iron per h.

The response of both mixed cultures to changing inflow rates of ferrous iron were very similar and reflected the same observations. One vessel is used to illustrate the changing population dynamics within both the mixed cultures. Overall performance of iron oxidation against time (fig 4.36) was similar to the performance for the L.ferrooxidans monoculture (fig 4.32) including the

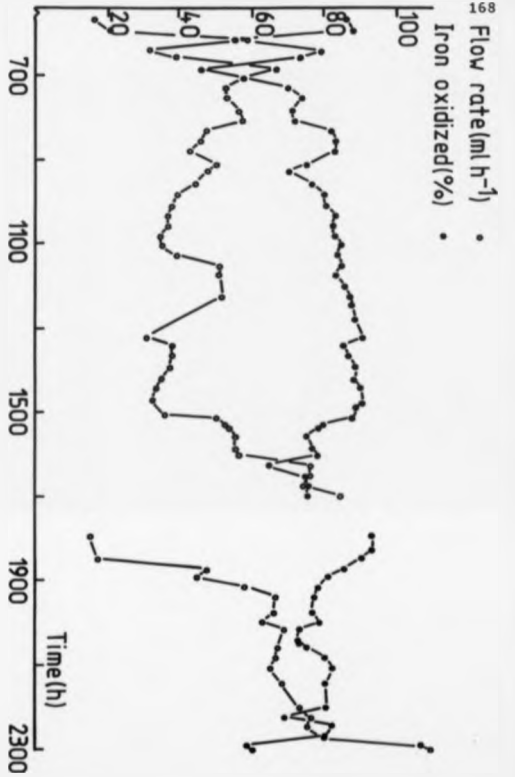


Fig 4.35
Flow rate of medium and % iron oxidised against time during continuous culture of T.ferrooxidans on ferrous iron. Growth was at 32°C in magnetically stirred vessel with ES, kept below pH1.6 during oxidation and containing 50mM ferrous iron substrate. Culture volume of 510ml aerated at 50ml per min. Data prior to 500h not plotted due to discovered inaccuracy in measured flow rate. Break in plot indicates restart in the indicated flow rate.

maintenance of ferrous iron at a limiting concentration within the vessel. This was probably due to the appearance within the mixed culture of cell aggregates after some 800 h of total continuous operation of the vessel, which undoubtedly gave the Leptospirillum a competitive advantage.

Microscope counts were made of the cultures prior to mixing. Counts were averaged for L.ferrooxidans at 2.8 x 10^8 organisms per ml and for T.ferrooxidans at 8.41 x 10^8 organisms per ml. After several volume changeovers when mixed the total organism count averaged 4.34 x 10^8 organisms per ml.

Four samples were taken from the mixed culture. At D equal to 0.023 h^{-1} the estimation of relative concentration of Leptospirillum cells based on microscopic examination was greater than 95% of the total cell number. Based on differential inhibition of the cell suspension from the sample the calculation was of 93.8% Leptospirillum and 2.6% Thiobacillus, a ratio of 36.1 to 1. (The first sample from the alternative mixed culture at a similar value of D had a "microscopic" estimation of 90% Leptosirillum, which on calculation was 91% Leptospirillum, 12% Thiobacillus). The cell ratios based on differential inhibition are given for all four samples in table 4.6. (The arithmetic use of the inhibition data is outlined in appendix 4).

After the first sample the flow rate was increased and resulted in a decrease in ferrous iron oxidized which was initially not halted by reducing the flow rate (fig 4.36). At 680 h the vessel was only 20% oxidized. Microscopic examination noted that the remaining cells were almost exclusively Thiobacillus ie the Leptospirillum had been washed out. Recovery was allowed prior to sampling to avoid sacrificing the culture due to the expected small cell yield at 20% iron oxidized. The protein concentration had decreased but the estimation of mesophile numbers was of near parity (table 4.6). The Leptospirillum population had recovered and was at least equal to that of the Thiobacillus. Microscopic

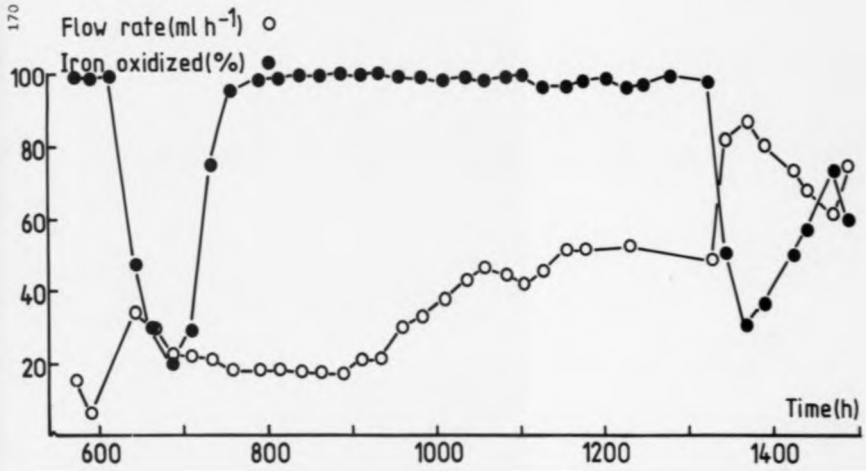


Fig 4.36 Medium flow rate and % iron oxidized against time during continuous culture on ferrous iron of a mixed culture of T.ferrooxidans and L.ferrooxidans. Enriched salts medium with 50mM ferrous iron substrate was kept below pH1.6 during oxidation and magnetically stirred at 32°C. Aerated at 50 ml per min, operating volume was 455ml. (Initial data not plotted due to discovered inaccuracy in measured flow rate).

examination agreed with this estimation. Subsequently the Leptospirillum formed aggregates during which complete oxidation in the vessel was maintained (fig 4.36). Establishing a steady state, protein content showed an increase of several fold whilst the oxidation studies indicated an absence of Thiobacillus cells (table 4.6). As this indicated dominance of Leptospirillum at low dilution rate, flow rate was increased in order to try and effect a Thiobacillus dominated culture as had been transiently observed prior to Leptospirillum aggregating.

TABLE 4.6

Percentage iron oxidation in cell suspensions from continuous culture. D (h^{-1}) and protein (mg per ml) are for the mixed culture. Values are for 100mM copper and 35mM nitrate (25mM-sample 1). (Calculations outlined in appendix 4).

Sample	1	2	3	4
T.ferrooxidans				
copper	78	89	100	100
nitrate	46	33	30	43.7
L.ferrooxidans				
copper	32	49	45	50.3
nitrate	100	64	64	91.5
Mixed culture				
copper	32	77	44	76.8
nitrate	95	57	76	89.3
Dilution rate	0.023	0.048	0.041*	0.112*
Protein	10.8	6.4	27.4	20
Cell ratio, Leptospirillum to Thiobacillus	36.1:1	1.21:1	L.f.	2.2:1

* Samples taken after observation of aggregating Leptospirillum cells in the mixed culture.

Iron oxidation up to 1200 h total operation was not affected by the increase in flow rate. The fourth sample at a dilution rate of 0.112 h^{-1} had a decreased protein content and an increased Thiobacillus content of approximately 37% (table 4.6).

Approaching a dilution of 0.18 h^{-1} the vessel was close to washing out. Oxidation was controlled by the flow rate comparable to the T.ferrooxidans mono-culture (figs 4.35,4.36).

4.5 DISCUSSION AND SUMMARY

Ferrous iron oxidation kinetics in cell suspensions were studied in order to provide organism specific values that could be related to observed growth. Values were calculated under conditions where rate increased proportionally to the protein (cell) concentration used (fig 4.5). Oxidizing activity was apparent in cell suspensions of all the measured strains (4.1). Kinetic measurements were statistically derived from linear forms of the Michaelis-Menten equation. Values of apparent K_m for each linear plot are summarized in table 4.7. These could be compared with previously published figures, though they were specific to the experimental conditions (4.2.1) particularly the pH of 1.7.

Iron oxidation by T.ferrooxidans was competitively inhibited by hydrogen ion concentration (fig 4.1) in contrast to the non competitive inhibition observed by Kelly and Jones (1978). This was for derivatives of the same strain. No major differences existed in use of equipment, substrate or protein concentrations, although one more pH value and three more (duplicated) determinations are presented here (fig 4.1; 2.4.1; Kelly and Jones, 1978). Similarly, differences appeared in apparent K_m figs for strain TH1 and both T.ferrooxidans and L.ferrooxidans.

The only previous kinetic study of strain TH1 used cells grown with 0.02% w/v yeast extract (Brierley et al, 1978).

Apparent K_m was reported as 7.3 mM ferrous iron with a V_{max} of 2.9 μ moles oxygen per min per mg protein. This value was considerably higher than the K_m of 1.03 mM, averaged over four determinations in this study , and not influenced by growth with yeast extract (table 4.7). The average V_{max} value was also less at 1.57 μ moles oxygen per min per mg protein but was close in the outside values. Brierley at al (1978) reported no loss of oxidizing activity for strain TH1 during storage at 4°C but this was contrasted with loss during actual experiments (fig 4.2). The presence of competitive inhibition by ferric iron was confirmed (4.2.4, fig 4.8). The K_i value of 2.59 mM with 10 mM ferric iron (table 4.8) was close to that of Brierley at al (1978) of 2.9 mM with 15 mM ferric iron. Therefore strain TH1 was found to have a higher affinity for ferrous iron, but a comparable response to ferric iron from what had been reported.

T.ferrooxidans (Yates and Nason strain) had a measured K_m in cell suspensions for ferrous iron of 0.70 $\pm$ 0.14 mM from six determinations (Kelly and Jones,1978). The value for a derivative culture, TFI-35 was 1.51 mM (DiSpirito and Tuovinen,1982). Strain DSM 583 (another derivative culture) in this study was closer to the latter value at 1.34 $\pm$ 0.16 mM. The alternative strain LA (of separate DNA homology,1.7.3) had a K_m of 0.85 mM (table 4.7). The response of the organism including the length of acceleration phases in the oxygen electrode was the same as that reported. Kelly and Jones observed competitive inhibition by ferric iron with K_i of 8-10 mM. This was confirmed but with a lower K_i of 3.10 $\pm$ 0.18 mM (4.2.1,1.7.1). (This value was though subject to the method of calculation). The response to pH (above) was in disagreement.

The relationship between K_m and K_i appeared to be constant over a spread of specific values (table 4.2) for DSM 583. Similar observations were made with strain ALV and (partially) strain TH3 (table 4.8). This ratio during inhibition by copper clearly distinguished between the two Thiobacillus strains in this study. No investigation

TABLE 4.7

Comparison of the apparent K_m values for ferrous iron by cell suspensions of acidophilic bacteria. Values (in mM) are derived from three alternative linear forms of the Michaelis-Menten equation. Standard deviation (for n-1) is for the number of experiments (shown in brackets). Values for V_{max} are determined from the double reciprocal plot in μ moles oxygen per min per mg bacterial protein. All cultures were grown chemolithotrophically except *, plus 0.01% w/v yeast extract.

Strain	Graphical Plot			V_{max}
	1/S vs 1/V	S/V vs S	V vs V/S	
T.f DSM 583	1.34±0.16(6)	1.44±0.61	1.27±0.16	1.66±0.77
T.f Strain LA	0.85(2)	-	-	-
L.f Markosyan	0.25±0.08(8)	0.15±0.07	0.26±0.08	0.49±0.13
Lep. et BC	0.31(1)	-	-	0.20
TH1	1.03±0.09(4)	2.05±0.63	1.06±0.06	1.57±0.48
TH1*	1.02(1)	-	-	1.54
TH3	0.45±0.11(4)	0.62ϕ	0.45±0.13	3.89 (3)
ALV	2.96±0.68(6)	3.56±0.73	1.06±0.80	0.74±0.13
LM2	1.525(1)	1.97	1.54	0.58
Sulfolobus	0.56±0.03(3)	0.695±0.29	0.60±0.3	1.34±0.26

ϕ S D not calculated due to scatter in values (4.2.4)

was made of the significance of this ratio as opposed to the actual constants.

L.ferrooxidans had the lowest K_m of 0.25 ± 0.08 mM (table 4.7). This was at the lower end of a previous

TABLE 4.8

Comparison of measured K_i for inhibitors of ferrous iron oxidation in cell suspensions of acidophilic bacteria. Figures (mM) are averages. With greater than two determinations (number in brackets) standard deviation is shown. Concentration of ferric iron used was 10 mM except * 25 mM. Selective values with 200 mM copper are also shown. The ratios of K_i to apparent K_m values (table 4.7) are shown with standard deviation.

Strain	K_i(Fe)	K_i/K_m	K_i(Cu)	K_i/K_m
T.f DSM 583	3.10±0.18(4)	2.45±0.23	178.7	127
T.f Strain LA	2.32(2)	2.745	178.2	318.2
L.f Markosyan	37.9/38.2*	128.9/106.1	uncompetitive	
TH1	2.59(1)	2.73	-	-
TH1 (+YE)	2.78(1)	-	-	-
TH3	1.89±0.39(4)	4.18±1.23	-	-
ALV	1.02±0.12(5)	0.34±0.07	-	-
Sulfolobus	1.71	2.87	-	-

determination of 0.5 ± 0.3 mM (Eccleston et al,1985). Ferric iron was confirmed as a competitive inhibitor of iron oxidation with good agreement in the calculated values of K_i, 37.9/38.2 mM (table 4.8), 33 ± 13 mM (Eccleston et al,1985). Of the mesophiles the greater sensitivity of L.ferrooxidans to copper was confirmed but shown to be uncompetitive in the form of inhibition (4.2.6,fig 4.13).

The thermophiles, previously unmeasured gave a range of kinetic values (tables 4.7,4.8). All the strains were subject to inhibition by ferric iron. This was competitive for strain TH1 (fig 4.8), strain TH3 (fig 4.9) and Sulfolobus BC65 (fig 4.11). Strain ALV only indicated

competitive inhibition by ferric iron at the higher concentrations of ferrous iron substrate. The curved plot of inhibited ferrous iron oxidation suggested that mixed forms of inhibition were operating in this acidophilic organism (fig 4.10). Both kinetic constants appeared to be related in strain ALV, supporting the observation with T.ferrooxidans. above.

The reactions of the thermophiles were strain specific with regard to retention of activity, presence of acceleration phases and deviation from maximum oxidation rate prior to oxygen exhaustion (4.2.1) in the oxygen electrode.

Strain specificity in relation to the influence of ferric iron was observed in batch culture. The length of lag phase was increased in growth curves of T.ferrooxidans as the ratio of ferric to ferrous iron initially present was increased to 5:1 (fig 4.15). In comparison, at this ratio L.ferrooxidans was not influenced (fig 4.16) yet strain ALV consistently failed to grow (fig 4.20). During iron oxidation strain ALV never displayed linear oxygen uptake, in the oxygen electrode, Warburg manometer or (logarithmically) during growth. The K_m for strain ALV in batch culture was close to 8mM ferrous iron. The only organism more sensitive to ferric iron during growth was strain LM2 which progressively failed during culture with 32 mM ferric iron intially present (fig 4.22). This strain had no comparable K_i measurement, 4.2.4. Therefore the extremes of unaffected growth and no growth above were for the organisms with the highest and lowest measured values respectively of K_i for ferric iron (table 4.8).The remaining organisms displayed inhibited oxidation in batch culture between the above two extremes.

Mixed cultures of T.ferrooxidans and L.ferrooxidans were studied under altered flow conditions and compared with pure cultures (4.4.4). Eccleston et al (1985) noted a critical dilution rate (D) of 0.1 h^{-1} for L.ferrooxidans with 100 mM ferrous iron input concentration. With 50 mM ferrous iron input, L.ferrooxidans did appear to be

washing out initially at D greater than 0.1 h^{-1} (fig 4.32). This was extended subsequent to cell aggregation concentrating biomass in the medium (figs 4.33,4.34). Thiobacillus cultures always retained residual ferrous iron in solution. In comparison the mixed cultures had high oxidation levels at low D. Sharp increases in inflow rate at this low D decreased percentage oxidation and appeared as a selective pressure for Thiobacillus cells. The balance was altered by aggregation of the Leptospirillum. At a D of 0.18 h^{-1}, the ratio of Thiobacillus to Leptospirillum increased (table 4.6). This was evidence for the greater affinity of Leptospirillum for ferrous iron, at low D where % oxidation was consistently higher and the greater growth rate of Thiobacillus, which increased in numbers proportionally to Leptospirillum at high D despite the inherent biomass concentration associated with aggregation.

Ferric iron production was related to protein concentration over time during retention of aggregated biomass (figs 4.25, 4.26). With 50 mM ferrous iron inflow the maximum dilution rate achieved was 0.17 h^{-1}. The peak production was 490 mg ferric iron per h. This contrasted with the Leptospirillum culture above (where no attempt at biomass retention was made) where productivity at D of 0.125 h^{-1} peaked at 140 mg ferric iron per h and protein concentration was an order of magnitude less.

In continuous culture with strain ALV (4.4.3) the maximum "yield" was 44 mg ferric iron per h. The percentage oxidation never rose above 50% iron oxidised and fell proportionately against dilution rate (fig 4.31). (These plots were not measured at true steady states). As measured in cell suspensions V_{max} for strain ALV was consistently greater than L.ferrooxidans (table 4.7). This suggested that level of oxidation and particularly decline with dilution rate was affected by other parameters. The sensitivity of strain ALV to ferric iron in batch culture and it's very low K_i figure indicated this as the dividing factor between the strains.

A T.ferrooxidans culture, kept at a pH at which jarosite did not precipitate produced 127.2 mg per h ferric iron at D of 0.12 h^{-1} (fig 4.30). Investigating the performance of a Bacfox type process the control vessel, without precipitates produced 131 mg per h ferric iron at D of 0.13 h^{-1} but with precipitates present, flow rate and therefore production were increased to 352 mg per h ferric iron at D of 0.23 h^{-1} (4.4.2). By increasing the internal surface area, though operating volume was greatly reduced, ferric iron production was measured at 602 mg per h ferric iron at D of 0.43 h^{-1}. (It was assumed that ferrous iron oxidation was complete and yielded ferric iron in the expected 1:1 ratio). Due to the instability of this system the sustainable production was probably less, at between 500 mg and 550 mg per h (4.4.2).

The kinetics of continuous culture of T.ferrooxidans were found to be complex and affected by product inhibition (Jones and Kelly, 1983). Jones and Kelly noted several points, relevant to the above data. The data above was not generated to measure kinetics in continuous culture as much as to observe whether the inherent kinetics that would be produced during growth under set imposed conditions could be predicted/explained by cell suspension kinetic data. The degree of product inhibition was predicted to depend on substrate concentration and D (Jones and Kelly, 1983). Here, with constant 50 mM ferrous iron, D became the important parameter. Jones and Kelly stated that smaller concentrations of ferric iron increased the level of residual substrate as D increased. It was expected that this increase in residual substrate concentration might be negligible where K_s (K_m) was small in the absence of inhibition. That is the oxidation could approach 100% or be little affected by values of D. Alternatively for a small K_p (K_i) inhibition would be experienced even at small concentrations of ferric iron in the medium.

Of the acidophiles, in this study L.ferrooxidans had the lowest K_s and highest K_i. During comparison with T.ferrooxidans, oxidation levels continuously approached 100%. Batch growth curves indicated no development of

product inhibition at similar substrate concentrations. Oxidation was unaffected by D until approaching 0.1 h^{-1}, the observed critical point (Eccleston *et al*, 1985) and predicted by doubling times observed in batch growth. *T.ferrooxidans* had a much lower K_i and an increased value for K_m (tables 4.7, 4.8). Iron oxidation never went beyond 95% at low D and steadily declined as the value of D increased. In mixed cultures oxidation levels greater than 95% coincided with cell ratios in favour of *Leptospirillum*. Strain ALV was incapable of complete iron oxidation in continuous culture (fig 4.31). It had a V_{max} greater than *L.ferrooxidans* in cell suspension but the lowest of all measured K_i values. The observed decreases in oxidation level were similar to those expected during forms of product inhibition.

The significance of the kinetic values was altered by biomass retention. Enhancement of *Leptospirillum* oxidation via it's physiological ability to aggregate (fig 4.24) increased the likelihood of it's being able to compete in natural conditions with *T.ferrooxidans*, under high flow conditions. The question was open as to the ability of these aggregates to form with iron precipitates and oxidise in directly comparable conditions to *Thiobacillus*.

In conclusion, the oxygen electrode was used to measure iron oxidation kinetics of a range of acidophiles. The response of the cells and measurements in the electrode were consistent and organism dependant. Distinctive growth curves in batch culture followed the pattern of kinetic measurements, particularly the values of K_i. This predicts that strain LM2 has a lower K_i value than any of those measured. That these measurements were predictive of growth responses was supported by continuous culture. Competition between mesophiles was relative to cell suspension kinetics as was the poor response of strain ALV during continuous growth. The ability of continuous culture to reflect the same actual specific kinetic values as opposed to an expected growth response, was not tested. That growth could be enhanced beyond expected limits by using biological as opposed to physico-chemical features

was shown with L.ferrooxidans. The relevance to natural systems was not tested.

No direct implication could be drawn for observed closeness of K_i and apparent K_m values. The order of the ratios followed the order in size of K_i values for T.ferrooxidans,L.ferrooxidans and strain ALV. Wider study would indicate if this was consistent with all strains although the limited evidence of table 4.8 suggests not. In addition evidence would be required to relate an observable physiological feature to the value of the K_i/K_m ratio. The relevance of this could be tested on a range of T.ferrooxidans strains. Strain LA had a smaller increase in lag phase in batch culture due to ferric iron than DSM 583 (fig 4.14), but had a smaller value of K_i. In contrast the K_i/K_m ratio was greater for strain LA. This contrasts with the conclusion above that greater effect of ferric iron would be seen for a smaller value of K_i. Testing T.ferrooxidans strains under the same conditions would also finalise differences in reported kinetic values of these strains (aggravated by the non-distinction of these strains into their observed separate genetic groupings). Subsequent growth measurements would hence answer the question of K_i/K_m relevance and whether kinetics were "strain" or genetic-dependant. The answer would strengthen the power of the oxygen electrode as a predictive tool if K_i/K_m was found to be significant. However, this study already indicates the usefulness of this tool by comparing a range of iron-oxidising acidophiles and relating the significance of measured values to growth conditions.

5 COMPARATIVE INVESTIGATION OF RESPIRATORY CHAINS

5.1 INTRODUCTION AND METHODS

Several of the acidophilic strains were comparatively studied with respect to their respiratory chain components. The cells, harvested from batch cultures were washed in acidified water, pH 2 and frozen as bulked pellets (2.5). T.ferrooxidans was used as the standard organism. This established an experimental standard of spectra for a respiratory chain of known composition and as a control of the experimental methods.

Growth media were kept as simple (and autotrophic in composition) as possible. However, as an aid to biomass production strain TH1 was cultured with a growth supplement of 0.01% w/v yeast extract. To increase biomass yield of Sulfolobus BC65, a ground mineral was used instead of ferrous iron as the energy source. This also increased the ease of handling of batch cultures at 65°C by greatly decreasing the size of required bulk volume relative to ferrous iron cultures. The strains used were T.ferrooxidans DSM583, L.ferrooxidans Markosyan, Sulfolobus BC65 and strains TH1 and ALV. In general, the media composition reflected the chemolithotrophic physiologies studied throughout this project. Under these conditions the experimental measurements were largely qualitative. The effect of nutritional supplements on either the qualitative or quantitative composition of the respiratory chains was not investigated. Neither was the response of the respiratory chain to switches of energy source, such as iron to sulphur or in switches of physiology such as chemolithotrophic to mixotrophic. (These changes did though result in morphological size differences between autotrophic moderate thermophile cultures and those supplemented with yeast extract). This crude qualitative comparison of electron transfer

components was on freeze-thawed pellets (0.5 g to 1.2 g wet weight) resuspended in 50 mM beta-alanine sulphate buffer at pH 3.5 (methods and conditions,2.5). These suspensions were kept on ice or at 4°C for the duration of the experiments.

5.2 WHOLE CELL SPECTRA

Cell suspensions showed little evidence of disruption on freeze-thawing. Microscopically, the thawed suspensions appeared as dense preparations of whole cells. Each thawed suspension was optically scanned, for wavelengths from 400 nm to 700 nm in a spectrophotometer, at room temperature. The resultant traces of absorbance maxima were prone to interference with background signal noise. Background signals were in part alleviated by further dilution of the thick suspensions in buffer. This interference was probably caused by using whole cell suspensions. Interference was further reduced by decreasing the measured sensitivity of the scans. Balance had to be made in that low absorbance was enhanced by increased sensitivity to differentiate between absorbance maxima. Therefore, spectra are often composites of two separate scans measured at different sensitivities. (Appropriate scale bars are presented on the corresponding figures). Lack of signal detail in the Soret region was unresponsive to this physical manipulation and was generally unrecorded. Each strain was scanned optically for a difference spectrum between reduced and oxidized samples. (Experimental details, 2.5 and appropriate figure legends.)

5.2.1 T.ferrooxidans standard

Room temperature whole cell difference spectra of T.ferrooxidans displayed only three major absorbance peaks, at wavelengths of 598 nm, 552 nm and 524 nm (fig 5.1). Though showing characteristic signals for this organism, these spectra lacked details in resolving separate absorbance maxima, known to be detectable by optical means. This resolution of signals due to the

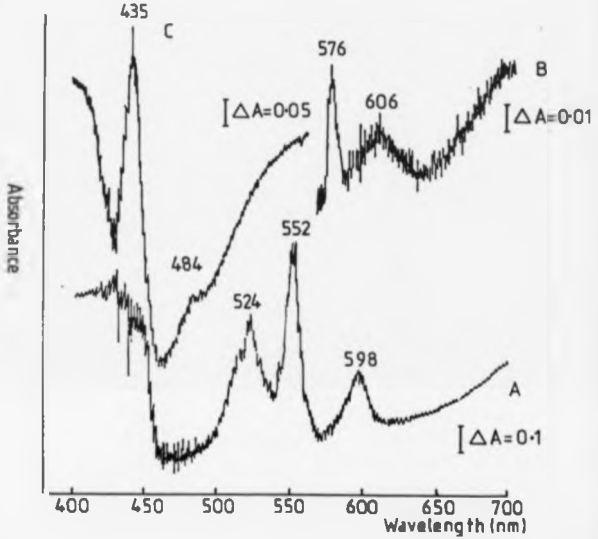


Fig 5.1
Optical spectra for whole cell suspensions at room temperature of T.ferrooxidans(trace A) and Sulfolobus BC65(traces B and C). Samples were scanned in glass cuvettes of 1cm pathlength at wavelengths from 400nm to 700nm in a dual beam spectrophotometer. Major absorbance peaks are noted and the scale bars indicate the absorbance scale(cm^{-1}) for each (part) trace. Traces were measured as reduced minus oxidised difference spectra, against an oxidised only baseline. Reduction was achieved by adding grains of dithionite to the sample. Cell concentration was 16.7 (T.ferrooxidans) and 7.58(Sulfolobus BC65) mg per ml protein in suspension.

presence of other absorbing components, not initially detected, was provided by low temperature spectrophotometry. Cell preparations were scanned at the temperature of liquid nitrogen, (-196°C). The resultant crystallization of the cell sample increased the path length of the light beam through the sample cell, due to reflection and refraction processes. This resolved signal masking where multiple absorbance was present in a relatively narrow wavelength range. Calculation of the relative concentrations of the individual absorbing "species" could not be made. Calculated on the basis of the generated signal height, this value was dependant on path length of light beam through the sample. Nominally, 1 cm, this path length became unknown and unmeasurable on freezing the sample. Neither could samples be presumed to crystallize identically for a comparison of concentration in empirical (rather than absolute) terms. Additionally, for known signals, absorbance maxima appeared at slightly shifted wavelength values recorded at -196°C rather than at room temperature. The temperature enhanced spectrum for T.ferrooxidans was almost identical to published results (Fig 5.2 cf Ingledew,1982). The signal at 597 nm was followed by two small shoulders at 585 nm and 557 nm. The latter signal was previously taken as indicating the presence of b-type cytochromes, whereas that at 597 nm was ascribed to a-type cytochrome. This spectrum (fig 5.2) was a recognisable signature for T.ferrooxidans, including evidence of more than one cytochrome-c, with signals at 552 nm and 548.5 nm. Whilst confirming the accuracy of the experimental method, this result also confirmed that absence of a (type of) component could not be inferred by lack of signal at room temperature alone (eg cytochrome b).

5.2.2 Comparative difference spectra - room temperature

Whole cell suspensions were optically scanned for the other four organisms. Each one was shown to provide a distinctive difference spectrum, at room temperature (figs 5.1,5.3). Requiring increases in measured sensitivity to effect a recordable scan of absorbance, these traces were increasingly prone to background signal noise. The

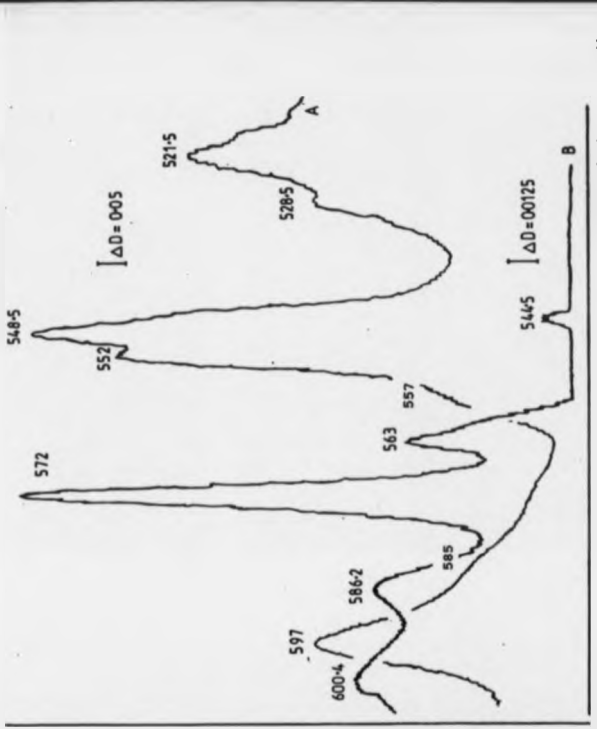


Fig 5.2
Optical spectra measured at-196°C for whole cell suspensions in a dual beam spectrophotometer of T.ferrooxidans(trace A) and Sulfolobus BC65(trace B). Samples were scanned at wavelengths from 500nm to 600nm in cells of nominal 1cm pathlength. Major absorbance peaks are noted, the horizontal scale being slightly different for each trace. The scale bars represent the sensitivity (as deflection voltage, mV, across the chart trace). Traces were measured as reduced sample (with sodium dithionite) minus oxidised sample for cell concentrations of 16.7 (T.ferrooxidans) and 7.58 (Sulfolobus BC65) mg per ml protein in suspension.

absorbance measured was generally in broad signals, lacking in definition, yet of an overall individual profile. (Figures represent absorbance traces with reduced signal noise). Immediate differences in profile were obvious between the organisms. Contrasting with T.ferrooxidans strains ALV and TH1 displayed strong similarities with each other (fig 5.3). Broad absorbance maxima were centred at 604 nm, 564 nm and from 528 nm to 530 nm for both strains. Absorbance data of known mitochondrial components attributed absorbance at 604 nm to cytochrome aa_3. Indication of cytochrome aa_3 was also given for Sulfolobus BC65 by absorbance at 606 nm (fig 5.1). No such indications were apparent for either the Thiobacillus or the Leptospirillum. Strain BC65 had only one other absorbance maximum in the visible region, at 576 nm. It was the only strain for which absorbance could be recorded in the Soret (400 nm-500 nm) region under these conditons. These peaks were at 484 nm and 435 nm (fig 5.1). The absorbance of the L.ferrooxidans suspension consisted of a broad alpha band centred on 578 nm and two smaller signals at 556 nm and 534 nm (fig 5.3).

These spectra indicated success in measuring absorbance of the respiratory chain components for this range of iron oxidizers. Attribution of the type of components could be made based on absorbance wavelength but these identifications were not confirmed experimentally (by isolation and biochemical identification). Based on results for T.ferrooxidans of increased resolution, and initial indication of significantly different respiratory chain profiles, absorbance was re-measured at low temperature.

5.2.3 Difference spectra - ultra low temperature

Frozen at -196°C L.ferrooxidans suspension displayed no absorbance at 578 nm (cf room temperature spectrum) even allowing for temperature shifts in absorbance maxima. Two separate maxima were present at 585 nm and 573 nm. The twin nature of the peaks suggested a splitting of the broad 578 nm signal into its separate component signals (fig 5.4). This resolution of broad signal was repeated

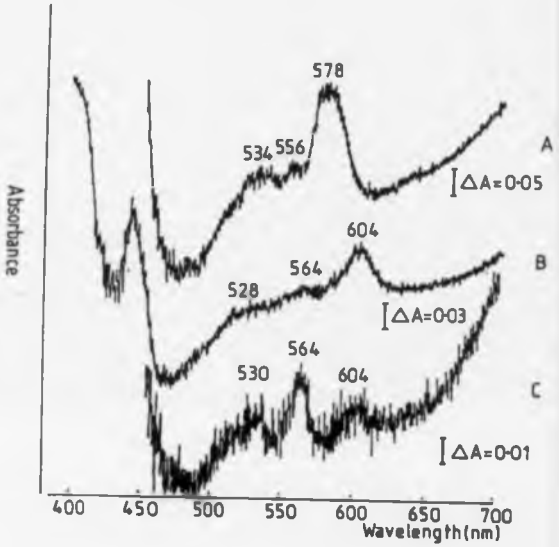


Fig 5.3
Comparison of optical spectra measured at room temperature in a dual beam spectrophotometer for whole cell suspensions of L.ferrooxidans(trace A), strain ALV(trace B) and strain TH1(trace C). Traces were measured as reduced minus oxidised difference spectra in glass cuvettes of 1cm pathlength and scanned at wavelengths of 400nm to 700nm. Definition in the Soret region (below 500nm) was affected by background signal noise and is not fully plotted. Samples were reduced with sodium dithionite. Wavelength of major peaks noted, scale bars indicating the relative sensitivity as absorbance change(cm^{-1}) of each measurement. Cell concentration was 8.5(L.ferrooxidans). 16.75(strain ALV) and 15.3(strain TH1) mg per ml protein in suspension.

by two small signals at 555 nm and 551.5 nm (in comparison to one room temperature signal of 556 nm). In addition previously unresolved signals stretched in a series of small peaks between 525 nm and 551.5 nm (fig 5.4).

The nominal similarities in absorbance profile for strains TH1 and ALV were repeated at low temperature. The respective spectra had an alpha band absorbance at between 601 nm and 603 nm. Overall the shape for each spectrum followed the same pattern with a shoulder at around 590 nm and a major signal at between 561 and 563 nm. Recorded scale, ie sensitivity used was greater for strain TH1 than strain ALV probably due to low cell concentration (fig 5.4). As a result strain TH1 indicated no further absorbance in the shorter wavelengths between 550 nm and 500 nm. In contrast strain ALV displayed several weak signals at these shorter wavelengths.

Sulfolobus BC65 absorbed at 600.4 nm with the major absorbance peak at 572 nm. An additional signal (to those at room temperature) was apparent at 586.2 nm (fig 5.2). This appeared to be the separation of mutually masked signals. Further visible region absorbance was observed at 563 nm and 541 nm.

(Absorbance below 500 nm at -196°C was not investigated for any of the strains).

5.2.4 Carbon monoxide binding - evidence for oxidase

Alteration of optical spectra, due to the addition of carbon monoxide to samples, was used as a probe for oxidase activity. Ligand binding of carbon monoxide (CO) was generally coincidental with the ability to bind oxygen. Cell samples reduced with sodium dithionite were used as blank controls in difference spectra against reduced cells gassed with pure CO (2.5). Spectra at room temperature gave no absorbance in the visible region. Each strain did though display non-specific signals in the Soret region (except L.ferrooxidans). These were composed of troughs and peaks in the baseline, at varying wavelengths depending on strain. This inferred binding of CO by four out of five strains. Identification of the bound components (identified by absorbance wavelength)

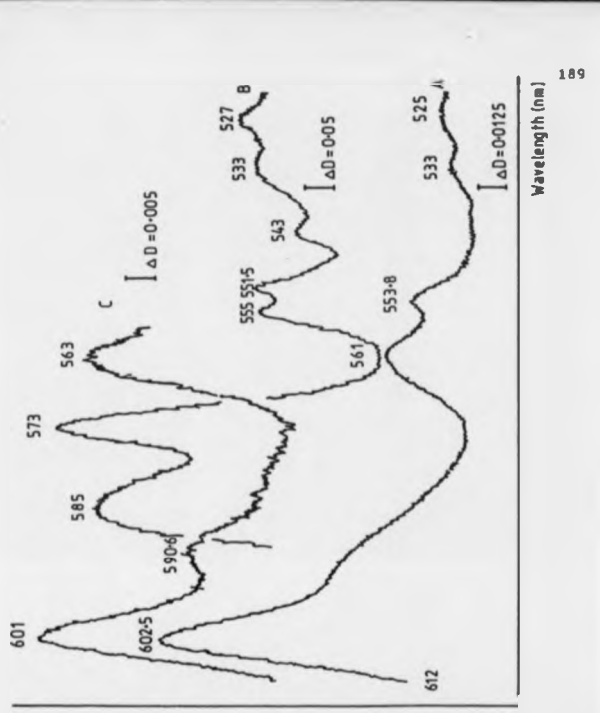


Fig 5.4
Comparison of optical spectra measured at -196°C in a dual beam spectrophotometer for whole cell suspensions of strain ALV(trace A), L.ferrooxidans(trace B) and strain TH1(trace C). Measurement was of reduced minus oxidized samples in cells of nominal 1cm pathlength. Cell reduction was with sodium dithionite. Horizontal scales differ slightly between traces and major absorbance peaks are noted. Scale bars are of deflection(mV) setting of the chart recorder (equivalent to sensitivity). Cell concentration was 8.5(L.ferrooxidans), 16.75(strain ALV) and 15.3(strain TH1) mg per ml protein in suspension.

based on shifts in previously observed absorbance maxima could not be made given the lack of signals in the visible region. The cell suspension of L.ferrooxidans precipitated out, preventing the test for CO binding. Resuspension was insufficiently sustained to record the appropriate optical spectrum. The supernatant of the settled suspension was red. Previously, suspensions of L.ferrooxidans allowed to settle on storage had easily resuspended without any colour change in the liquid (acidified water). Colouration of the liquid suggested some reaction that had physically altered the cells. Resuspension was attempted in the presence of a surface active agent, Triton X 100. This caused frothing on bubbling CO, but did not maintain resuspension long enough. Investigation of CO binding on frozen sample was unsuccessful, as was an attempt with T.ferrooxidans (and was not attempted on the other strains).

5.3 OPTICAL SPECTRA - CELL FREE EXTRACT

Investigation of the respiratory chains identified L.ferrooxidans and Sulfolobus BC65 as potentially the most novel organisms (biochemically). Both displayed major absorbance at 578 nm and 576 nm at room temperature not immediately attributable to known respiratory compounds even with enhanced resolution of signals at -196°C. This signal, nominally similar for either organism was chosen for further study/identification. Investigation centred on L.ferrooxidans due to ease of handling during bulk growth of this organism compared to the thermophile. Simultaneously L.ferrooxidans was to be tested for the presence of rusticyanin (or similar enzyme) previously identified in T.ferrooxidans (1.7.2). To isolate any such compounds into solution and in an effort to reduce signal noise in optical spectra, samples were prepared as cell free extract. T.ferrooxidans was used as a control against unfamiliar experimental procedure. Preparation of cell free extracts was an adaptation of methods published for T.ferrooxidans (Cobley and Haddock, 1975; Cox and

Boxer, 1978) using a French pressure cell. Passage of the Leptospirillum through the pressure cell was difficult given it's tendency to stick. Both organism preparations were prepared in water pH 2, centrifuged and final extract prepared by ultra centrifugation (2.5.2).

5.3.1 T.ferrooxidans standard

Acidified cell free extract of T.ferrooxidans was discernibly blue (but very pale cf deep blue colouration of Cox and Boxer, 1978). Lack of colour was probably due to over dilution of the cell suspension and therefore to lack of the colour associated rusticyanin. Subsequent optical spectra appeared to confirm this statement,lacking absorbance at 590 nm. This broad absorbance band, distinctive for rusticyanin was masked in whole cell spectra. An apparent slight signal at 595 nm was not bleached by adding ferrous iron or dithionite and so was discounted. Addition of ferrous iron did produce two small signals at 550 nm and 520 nm, intensified by adding dithionite. These signals, speculated as cytochrome c by Cobley and Haddock, confirmed their results and also the experimental procedure (in all but original cell concentration).

5.3.2 L.ferrooxidans extract

Cell free extract of L.ferrooxidans in acidified water was a strong reddish-brown colour, similar to ferric iron. No absorbance was measured spectrophotometrically for ferric iron, using spent ferrous iron medium as a positive control. Against a water blank, oxidised extract at 30°C gave no absorbance at 590 nm. Absorbance consisted of a shoulder at around 575 nm and a broad signal centred on 533 nm. This latter signal narrowed and shifted to 535 nm on adding 10 mM ferrous iron. Major absorbance was also evident at 577 nm , assumed to be due to reduction by ferrous iron. Partial reduction of oxidised sample occurred after six days storage at 4°C. Such partially reduced samples, originally prepared at pH 2.46, were completely reduced on adding 4 M KOH. The absorbance of the subsequent alpha band was at 577.5 nm, similar to ferrous iron, above. L.ferrooxidans cell free extract did

not therefore indicate the presence of rusticyanin. It did show evidence of an acid soluble red pigment, responsible for absorbance at around 577 nm when reduced. Given that such acid solubility effected a crude purification of this absorbing species, as most respiratory proteins would be denatured, further investigation used acidified cell free extract.

Accurate room temperature scans were performed on fresh acid-extract to produce reduced minus oxidized difference spectra. The overall profile (fig 5.5) was of absorbances at 578 nm, 534 nm and 441 nm, in the Soret,confirming the assumption of reduction,above. (Low temperature spectra were not measured. That the signal at 578 nm was again a composite of two signals was therefore not determined). Difference spectra were comparable, using dithionite, ferrous iron or alkali. However, absorbance maxima were at slightly longer wavelengths with ferrous iron than dithionite, ie 580 nm, 539 nm and 443 nm.

5.3.3 Carbon monoxide binding

The Leptospirillum cell free extract was affected by CO. Addition of CO to the reduced cell in a difference spectrum, using dithionite, shifted the absorbance maxima to longer wavelengths. At 579 nm and 537 nm this shift was smaller than that observed with ferrous iron above. For identical samples (as protein concentration) the degree of absorbance was also altered by choice of reductant. That is signal height increased on reduction with ferrous iron, dithionite and dithionite and carbon monoxide together , indicating relative degrees of reduction. In conjunction with increased signal height, a shoulder at 418 nm was observed with dithionite and not ferrous iron. Further addition of CO produced a discrete signal at 416 nm. (That signal height and concentration of ferrous iron were related was not shown. An experiment to measure this and therefore extinction coefficient was unsuccessful).

Binding of CO was further investigated by addition to a fully reduced system. A scan of this system, plus and minus CO produced a difference spectrum. In the visible

Fig 5.5
Spectrum of cell free extract of L.ferrooxidans scanned at wavelengths from 400nm to 700nm in a dual beam spectrophotometer at room temperature. Scan was of reduced minus oxidised samples in glass cuvettes of 1cm pathlength. Sample was reduced on the addition of sodium dithionite. Scale bar is of absorbance change(cm^{-1}). Extract at pH2 was used at a concentration of 3.27mg protein per ml.

region broad signals were at 594 nm and 553nm (this on expansion was 556 nm and 548 nm). Signals in the Soret were at 437 nm and 416 nm (fig 5.6).

5.4 KINETIC MEASUREMENTS OF RED PIGMENT

Absorbance of the red pigmented acid extract, for the signal of interest, was between 578 nm and 580 nm dependant on conditions. The appearance of absorbance, at 579 nm by reduction with ferrous iron was measured against a time base. Maximum absorbance was achieved in five minutes. Measurement of this signal at wavelengths at 0.5 nm intervals centred the absorbance maximum more closely on 579.5 nm.The oxidation of the artificial substrate, ascorbate by the Leptospirillum pigment was tested in a Clark oxygen electrode. (Acid stable and reduced by ferrous iron, rusticyanin was capable of this oxidation, Ingledew, 1982). The final reaction volume contained 1 mM ascorbate, 0.1 mM of the redox enhancer* TMPD (NNN^1N^1 tetramethyl-p phenylene-diamine dihydrochloride) and extract at 109 µg protein per ml. However, no oxidation was displayed at any significant rate. (Slow oxygen uptake was observed on the further addition of ferrous iron (0.6 mM).Optical redox titration failed to give a satisfactory mid point potential for the protein content of the cell extract, absorbing at 578 nm. As an alternative, absorbance in the visible region was measured at varying concentration ratios of ferrous:ferric iron. At a fixed ferric iron concentration changing ferrous iron concentration created different redox potentials due to the ferrous/ferric couple. The development of a trend in signal height against the redox potential was inconsistent.

5.5 IDENTIFICATION OF PIGMENT VIA PROSTHETIC GROUP

Cytochromes were identified by their prosthetic (heme) groups. Optical spectra of acid cell free extract

*mediator in redox reactions

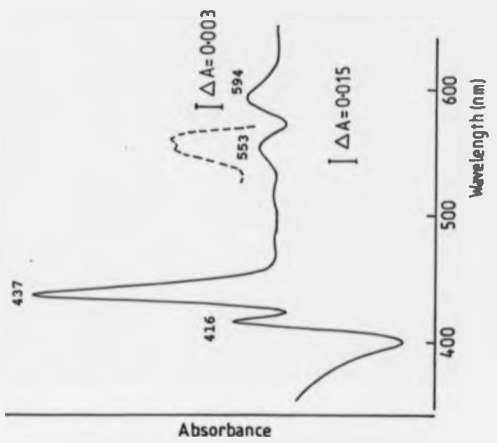


Fig 5.6
Difference spectrum caused by the addition of carbon monoxide to one half of a reduced system of reduced cell free extract of L.ferrooxidans optically scanned in a dual beam spectrophotometer. Scan measured at room temperature in glass cuvettes of 1cm pathlength. Scale bars are of absorbance change(cm^{-1}). Extract at pH2 was used at a concentration of 3.27mg protein per ml.

suggested one principal, red pigmented, absorbing compound of unknown identity. Binding of CO suggested a ligand, presumably a prosthetic group. Identification was based on the formation of alkaline pyridine derivatives by heme groups. Despite impurity of the sample, formation of a derivative was attempted. Particular attention was given to the acidity of the sample, to ensure final alkaline conditions. A trial derivation was prepared on used extract (contaminated with dithionite and other reductants). Final conditions were in 3 M pyridine anhydride and 0.2 M sodium hydroxide. A reduced minus oxidized difference spectrum was noisy but with distinct signals. Repeated with pristine extract, absorbance in the visible region was broad (fig 5.7). Major absorbance peaks were at 557 nm, 525 nm and 432 nm in the Soret. Additionally there was a small signal peak at 590 nm and a shoulder at 553 nm.

5.6 IDENTIFICATION OF UNKNOWN PIGMENT - DISCUSSION

L.ferrooxidans cells absorbed at 578 nm. This absorbance appeared associated with a soluble acid-stable red-brown pigment. This protein pigment was reduced both by ferrous iron and base addition, as well as by dithionite. It displayed binding of CO and the formation of a crude alkaline pyridine derivative. The wavelength absorbance of the latter was not characteristic of known heme prosthetic groups. A literature search was made of absorbance maxima related to respiratory/redox enzymes. One study identified a cytochrome b from a halotolerant Micrococcus (Mori and Hirai, 1968) with absorbance maxima at 574 nm, 537 nm and 418 nm (cf L.ferrooxidans, table 5.1). This similarity was discounted for several reasons. Absorbance of B-574 was on oxidized sample, for L.ferrooxidans, reduced sample. Leptospirillum was affected by CO, reduced B-574 was not. Finally, the pyridine hemochrome of B-574 was formed with absorbance at 557 nm (Mori and Hirai,1968), the red pigment,557nm.

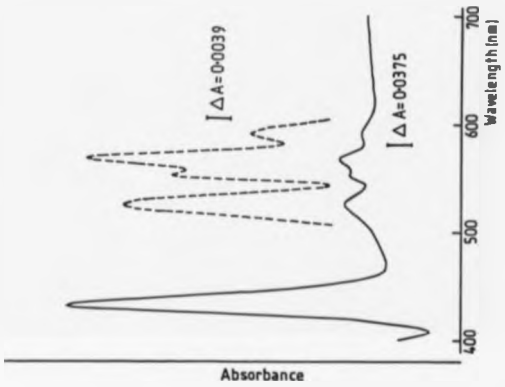


Fig 3.7
Difference spectrum of reduced minus oxidized samples of cell free extract of L.ferrooxidans (pH2) placed in suspensions with final concentrations of 3M pyridine anhydride and 0.2M sodium hydroxide and optically scanned in a dual beam spectrophotometer. Measured at room temperature, samples of 2.12mg protein per ml were scanned in glass cuvettes of 1cm pathlength. Scale bars are of absorbance change(cm^{-1}).

TABLE 5.1

Summary of the wavelengths of absorbance maxima for optical spectra of L.ferrooxidans. Major signals are underlined and shoulders indicated as (s).

(1) Difference spectra of whole cells at room temperature
578nm 556nm 534nm

(2) Difference spectra of whole cells at -196°C
585nm 573nm 555nm 551.5nm small signals 525nm to 551.5nm

(3) Difference spectra of cell extract at room temperature
578nm 534nm 441nm

(4) CO difference spectrum of cell-free extract
594nm 556/548nm 437nm 416nm

(5) Alkaline pyridine derivative of cell-free extract
590nm 567nm 551(s) 525nm 432nm

Alternative reports featured siroheme, an uncommon ligand binding prosthetic group associated with enzyme activity. Suggested as involved in electron transfer siroheme was shown as the site of interaction between electron transport and substrate. It was identified in two redox enzymes catalysing multi-electron transfers, sulfite reductases (irrespective of biological source) and nitrite reductases. Similarities existed between properties of siroheme proteins and red Leptospirillum pigment. This was inconclusive due to the former data being measured on pure enzyme or extracted prosthetic group and that for L.ferrooxidans lacking experimental detail, and being performed on crude samples.

However, purified spinach nitrite reductase absorbed at 573 nm and 388 nm. (Siroheme was shown causative of absorbance at 573 nm and 385 nm). L.ferrooxidans pigment (pigment -578) absorbed at 573 nm (at -196°C, table 5.1). The nitrite reductase shifted signals on reduction to characteristic wavelengths at 592 nm and 543 nm. Signals for pigment -578 were already measured in difference spectra. Shifts in wavelength on reduction were though enzyme dependent. One enzyme only shifted in alpha band

absorbance from 573 nm to 585 nm (Zumft, 1972). Whole cells of Leptospirillum absorbed at 585 nm when reduced -196°C table 5.1) whereas the oxidized sample of pigment -578 from cell extract absorbed with a shoulder at 575 nm (5.3.2). In comparison, bound with CO, nitrite reductase absorbed at 585 nm and 543 nm (in the visible region), pigment -578 at 594 nm and 548/556 nm. (Extracted sirohaem from any source absorbed plus CO at 593 nm). Finally sirohaem did not form typical pyridine haemochromogen (Murphy,Siegel,Tove and Kamin, 1974). Neither did pigment -578, being neither a, b nor c cytochrome type haem (according to general literature survey). The iron content of pigment -578 extract was not investigated.

The data were slightly different with sulphite reductases, typically absorbing between 582 nm and 587 nm (for microbial sources). Pigment -578 absorbed at 585 nm (at -196°C). These maxima shifted on reduction, to longer wavelengths than for nitrite reductases above, eg for E.coli the shift was 587 nm to 595 nm. In general for Enterobacteria, signals in the presence of CO were at 600 nm, 556 nm and 400 nm (pigment -578; 594 nm, 556/548 nm, 437 nm, 416 nm, table 5.1). But for Desulfovibrio desulfuricans absorbance with CO for sulphite reductase was at 593 nm and 550 nm, for the visible region (Siegel,Murphy and Kamin,1973).

Pyridine derivatives (0.1N NaOH and 2.5 M pyridine) of E.coli sulphite reductase showed absorbance at 553 nm, 520 nm (S) and 389/399 nm (extracted sirohaem 557 nm, 520 nm(s) and 401 nm, Murphy et al, 1974; cf pigment -578, table 5.1).

Therefore, in comparing data for pigment -578 the principal lack of detail was of measurement of cell free extract spectrum at -196°C. Table 5.1 shows signals at 573 nm and 534 nm common to both whole cells and cell free extract. The evidence of the former signal measurement being composite was not shown for cell free extract as was suggested for whole cells and was therefore speculative. Also, distinctive sirohaem absorbance was source

dependent, but in summary absorbance was given at 570-573 nm or 582-587 nm, shifting on reduction. Absorbance of pigment -578 nm in whole cells was at 573 nm and 585 nm (at-196°C) for reduced sample. But reduced siroheme proteins could absorb at 585 nm. Similarities in pyridine derivatives were slight but both were different from characteristic cytochrome prosthetic groups (even allowing for multiple signals for pigment -578 being due to contamination of sample). In contrast, reduced siroheme proteins had the unusual feature of no signal at 400-500 nm. Untrue for pigment -578 (table 5.1) measurement was not made at the shorter wavelengths characteristic of siroheme ie less than 400 nm. Finally, all siroheme proteins bound CO with absorbance at 590-600 nm, 550-560 nm and 395-400 nm. Pigment -578 absorbed at 594 nm, 548/556 nm, 437 nm and 416 nm (small signal).

5.7 SUMMARY

Respiratory chain compositions were different for all the strains (except between strains TH1 and ALV; 5.2.2,5.2.3). Components were not positively identified chemically. Estimation of relative concentrations was therefore difficult. All five organisms gave evidence of an oxidase, and except L.ferrooxidans of an a/aa_3 type cytochrome. Evidence of b and c type cytochromes in various concentrations were apparent for all five strains. The concentration of respiratory chain components apparently present gave no indication of any relation to rank relative to order of apparent K_m values. Both Sulfolobus BC65 and L.ferrooxidans displayed similar but unidentified absorbance which for the Leptospirillum had characteristics of a putative siroheme-containing protein.

This peak at 576nm was not observed in other strains of Sulfolobus. Grown heterotrophically S.acidocaldarius DSM639 gave difference spectra with peaks at 562nm and 587nm (Anemüller,Lübben and Schäfer, 1985). A closer value in strain 7 was given as 583nm (Wakagi and Oshima, 1987). Both studies used membrane fractions at pH5.5. With strain DSM639 pyridine hemochromogen derivatives gave peaks at 566nm and 587nm which were attributed to b-type cytochromes but not tested. The peak at 566nm compared with that at 567nm in Sulfolobus BC65. This raises the possibility of this unknown species being consistent in variant forms in other Sulfolobus species/strains.

6 THERMOPHILIC MINERAL LEACHING

6.1 CULTURE CONDITIONS AND THE ROLE OF IRON

Thermophilic bacterial leaching was known to occur and at rates greater than observed with T.ferrooxidans (1.8.3). Sulfolobus BC65 was studied physiologically with respect to some of the physico-chemical factors that influenced metal dissolution and the role of ferrous iron oxidation in the process. Routinely cultured on pyrite this thermophile was capable of extensive copper dissolution from both chalcopyrite ores and copper concentrates. The extent of dissolution differed with the source of mineral used. During shake flask culture with 1% w/v pulp density of a range of copper sulphides (all putatively predominantly chalcopyrite) the degree of copper release differed by greater than a factor of two (fig 6.1). The release from the most susceptible ore of 1.7 g per litre Cu was balanced by the release of 500 mg per litre Cu into the medium prior to inoculation. In comparison the release of copper from the Santiago copper concentrate differed in that pre-release of copper was less than 100 mg per litre. The shapes of the two release curves were similar. Chemical release of the copper gave no indication therefore of the susceptibility of an ore to bacterial dissolution. Observed also with certain pyrite ores no investigation of the mineralogy of these ores was made to determine the nature of their resistance or susceptibility to leaching.

Sulfolobus BC65 was isolated under carbon dioxide enrichment (Marsh and Norris, 1983) but the effect of it's limitation on sulphide leaching was not investigated. Batch cultures were grown in 500 ml vessels, with 1% w/v pyrite and gassed with 50 ml per min air containing 1% v/v carbon dioxide. On adding 4% w/v pyrite supplements to the grown cultures, the removal of carbon dioxide

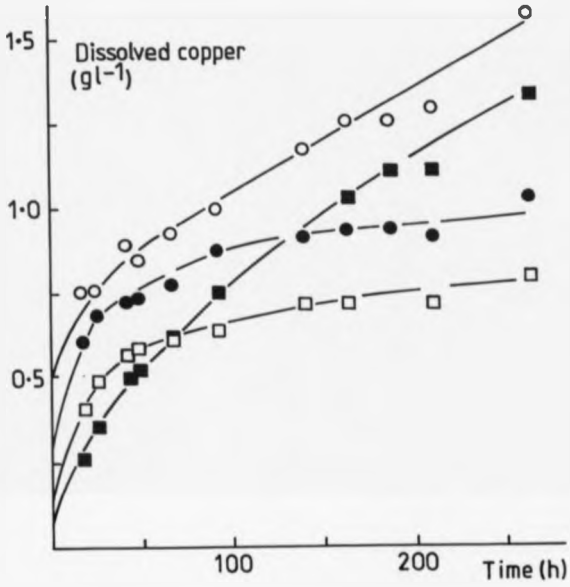


Fig 6.1
Comparison of copper release over time from different chalcopyrite ores during dissolution at 68°C with Sulfolobus BC65. Release was measured for ground sulphide ore(all passing a 200μm sieve) used at 1%w/v concentration in 100ml ES at pH2 shaken at 120rpm in 250ml flasks. Cultures were trickled with 1%v/v CO_2 in air. Ore samples were: from Santiago in Spain(■), from the Avoca mine in Eire(○), an Australian concentrate(●) and an unspecified sample(□). Inocula were 5%v/v from a pyrite grown batch culture. Samples were all measured in duplicate.

enrichment reduced the rate of pyrite oxidation (fig 6.2). With only 5 ml per min air, oxidation failed at 4 g per l Fe in solution. However, at 100 ml per min aeration although the rate was inhibited the extent of leaching approached that possible with carbon dioxide enrichment.

Release of metal (copper) from ores due to the effects of acid, heat and agitation increased with decreasing particle size. The rate and extent of bacterial dissolution also increased. In a shake flask experiment, of copper dissolution of different size fractions of a chalcopyrite ore, at 2.5% w/v, the leaching was greatest with the sub six-micron fraction (fig 6.3). Initially the rate of copper release from the largest size fraction was subject to an increased lag phase. This was consistent in duplicate culture as was the subsequently reduced copper release. The release of 5 g per litre Cu from the smallest size fraction was achieved at an initial pH of pH 1.3 demonstrating the ability of Sulfolobus BC65 to oxidize minerals at pH values inhibitory to T.ferrooxidans. The influence of particle size on extent and rate of leaching was more directly apparent with pyrite leaching at pH 2. (The preparation and measurement of different particle size fractions is outlined in Appendix 1). Size fractions of broader particle size range, -180 + 75 micron, -75 + 38 micron and sub 20 micron were added at 5% w/v concentration to overhead stirred fermenters containing identical cultures of Sulfolobus BC65 (fig 6.4). Estimations of iron release from the linear plots were of the order of 70, 100 and 160 mg per litre per h as the particle size decreased. During the sulphide oxidation the ferrous iron level remained constant at concentrations of up to 2 mM, except after the addition of the smallest size fraction. The release of 700 mg per litre Fe from the sub 20 micron fraction appeared to be exclusively in the ferrous form. Iron release from the other fractions (within an hour of addition) was less than 200 mg per litre. This ferrous iron was slowly oxidized during the following forty hours of growth. When stopped the pH value in the least leached vessel was pH 0.95 compared to pH 0.82 in the other two

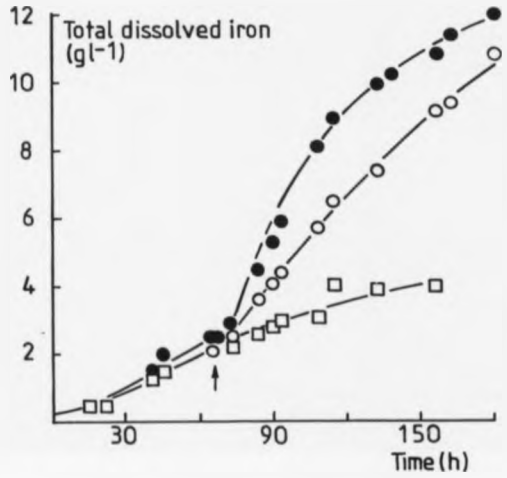


Fig 6.2
Effect of CO_2 on iron dissolution from pyrite during oxidation by *Sulfolobus* BC65 in batch culture at 68°C. Growth was in 500ml water jacketed vessels agitated by overhead paddled stirrers. Medium was 250ml 9K salts at pH2 with 1%w/v pyrite initially, a further 4%w/v pyrite(particle size -38+20μm) being added(shown by the arrow) on growth of the inoculum. Each vessel was sparged with 1%v/v CO_2 in air at 50ml per min prior to mineral addition. After the addition , vessels were sparged with 100ml per min air, only(○) plus 1%v/v CO_2(●) and plus 1%v/v CO_2 but at flow rate of 5ml per min(□).

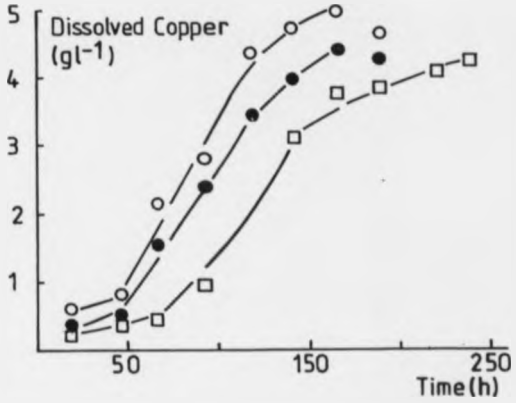


Fig 6.3
Comparison of copper release over time from different size fractions of the same (Santiago) chalcopyrite ore(22.5% Cu) during dissolution in batch culture of Sulfolobus BC65 at 68°C. Growth was in 100ml ES at pH1.3 in 250ml flasks shaken at 120rpm and containing 2.5%w/v ground sulphide. Medium was trickled with 1%v/v CO_2 in air. Size fractions(as calculated from cyclosizer operation) were -6μm(○), -9.3+6.8μm(●), >45μm(□) . (The printout of particle size analysis for these samples is given in figA1.1, samples 2,3,5 respectively).

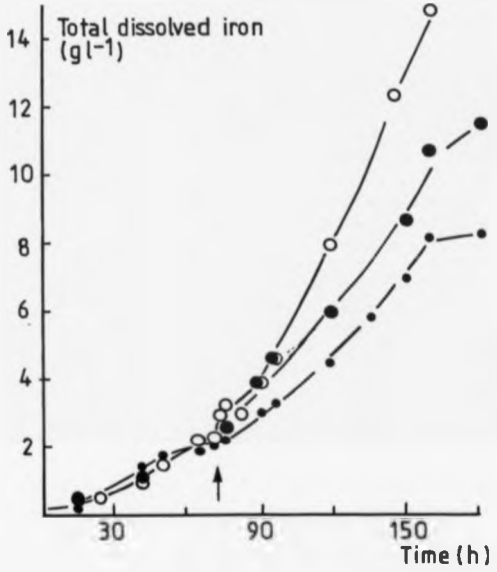


Fig 6.4
Comparison of the rate of total iron release over time with different particle size fractions of pyrite in batch fermenter culture of Sulfolobus BC65 at 68°C. Growth was in 700ml 9K salts at pH2 in 1 l stirred fermenters sparged with 350ml per min 1%v/v CO_2 in air. Initial growth was with 1%w/v pyrite of the smallest particle size prior to the addition(at arrow) of 5%w/v pyrite of sieved fractions at -180+75μm(●), -75+38μm(●) and -20μm(○).

vessels. This was indicative of the smaller concentrations of sulphate generated. In general, leaching was not observed to progress beyond pH 0.8.

The appearance of ferrous iron in solution, whilst apparently a function of particle size was also consistent, particularly with copper ores. The release of ferrous iron preceded release of the target metal, during oxidation of 5% w/v chalcopyrite (fig 6.5). Total soluble iron concentrations exactly matched the measured concentrations of ferrous iron until a peak was reached. As the ferrous iron concentration levelled off, at 3 g per litre, the total soluble iron concentration (not shown), increased. This indicated both the onset of ferrous iron oxidation and that no ferrous iron oxidation was intially occurring during it's release. Total iron levels began to fall, during the oxidation, suggesting the onset of metal precipitation. Copper solubilisation increased during the ferrous iron oxidation. No lags appeared in subsequent oxidation on the addition of supplements of 5g per litre ferrous iron. These concentrations, greater than that initially released from the ore were rapidly oxidized. Overall, the release of copper did not seem affected by the iron additions, although separate experiments with the same ore generally showed reduced copper dissolution averaged at less than 8g per litre copper in solution.

Ferrous iron was also released during thermophilic leaching of pyrite. With mineral additions to growing batch cultures, the ferrous iron release was instant. Averaging below 3g per litre this level fluctuated but remained relatively constant, only slowly reducing as the sulphide leach neared completion. This slow oxidation could be interrupted by adding fresh ferrous iron into solution. The addition of 16.7g per litre ferrous iron (300 mM) to a vessel with 3.5% w/v pyrite, total, resulted in rapid oxidation (fig 6.6). As the concentration of ferrous iron fell below 5g per litre, a second, much slower rate of oxidation was observed. Completion of oxidation coincided with limitation of sulphide leaching, masked by the precipitation of the high levels of ferric

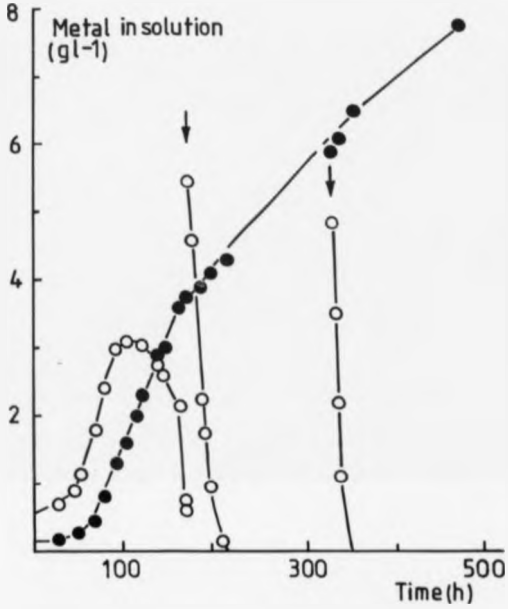


Fig 6.5
Concentration of ferrous iron(O) and total copper(●) during batch leach of chalcopyrite at 68°C with Sulfolobus BC65 and the influence of additions of ferrous iron. Growth was in 600ml 9K salts at pH2 in a 1 l fermenter gassed at 300ml per min 1%v/v CO_2 in air. Inoculum was 10%v/v from a pyrite grown batch culture. Substrate was 5%w/v Santiago chalcopyrite(-200μm particle size). Fresh non-sterile ferrous iron, $5gl^{-1}$ was added during the leach, shown by the arrows.

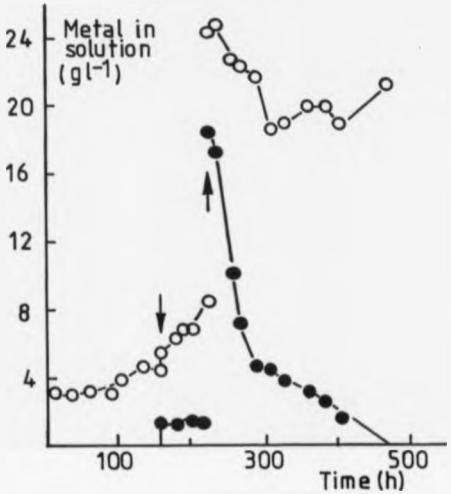


Fig 6.6
Concentration of ferrous iron(●) and total iron(○) during batch leach of pyrite at 68°C with Sulfolobus BC63 and the influence of additional ferrous iron. Growth was in 500ml 9K salts at pH2 in a 1 l fermenter gassed at 200ml per min 1%v/v CO_2 in air. Inoculum was 10%v/v, pyrite grown. After initial leach 2.5%w/v pyrite was added(-200 mesh. first arrow). During growth 300mM ferrous iron was added($16.7gl^{-1}$, second arrow).

iron.

At pH 1.4 the release of ferrous iron from copper ores was greatly extended, approaching 6g per litre, twice that averaged for leaches at pH 2 (fig 6.4). The addition of ferric and ferrous iron after the oxidation of the released ferrous iron, only marginally extended the levels of copper leached by a few hundred mg per litre indicating that the higher copper extraction of fig 6.4 was probably due to ferric iron generated from the ferrous iron supplement. However, the death of the culture on the addition of 10 ml panacide along with additional ferric iron, to a typical batch leach caused the reappearance of ferrous iron into solution, of up to 1.5g per litre.

During sulphide oxidation of 5% w/v copper concentrate (of 22.5% Cu) the highest concentration of ferrous iron released from this ore was 8.4g per litre at an initial pH of pH 1.4. At the observed peak of ferrous iron release, just over half of the 9.2g per litre copper released was in solution (fig 6.7). This percentage extraction, the best observed with this ore was achieved with a lean salts medium. Sulphide leaching was normally in 9K salts, but this experiment used enriched salts with 2.5 times the normal nitrogen concentration (media, 2.2.1).

6.2 DISCUSSION

Mineral dissolution at thermophilic temperatures, principally 68°C, was subject to a range of chemical and physical controls. The degree of metal dissolution, though extended by Sulfolobus BC65 compared to T.ferrooxidans, was limited by the source of mineral substrate (fig 6.1). Limitations on oxidation rate were caused by the influence of particle size and availability of carbon dioxide (figs 6.4; 6.2). Smaller particle size influenced the concentration of ferrous iron in solution at the start of leaching. The concentration of ferrous iron in solution from copper sulphide increased during leach experiments at pH 1.4 (figs 6.5; 6.7). The increase in acidity may have either influenced the leach

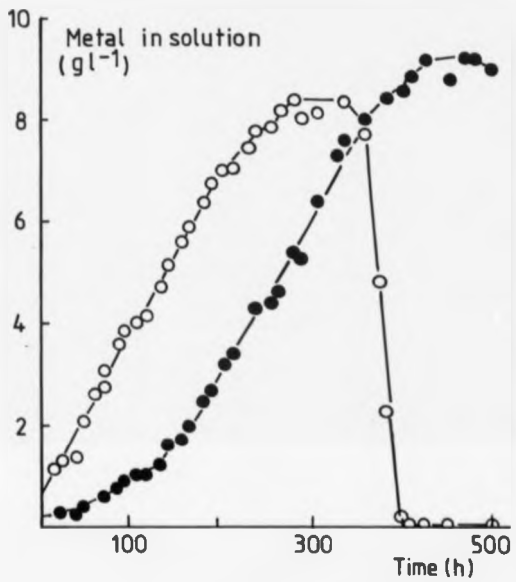


Fig 6.7
Concentration of ferrous iron(○) and total copper(●) in solution against time during batch leach of Santiago chalcopyrite ore(22.5% Cu, particle size less than 200μm) at 68°C with Sulfolobus BC65. Medium was ES with 2.5 times normal nitrogen concentration (but still significantly less than 9K salts, 2.2.1). Agitated by vertical paddled stirrer in 1 l vessel, medium at initial pH1.4 was aerated 300ml per min with 1%v/v CO_2 in air. Pulp density was 5%w/v. Inoculum was 10%v/v.

chemically, by attacking the ore, or biologically by preventing the onset of ferrous iron oxidation. Measurements of total soluble iron content during oxidation were of limited value due to the problem of ferric iron precipitation. However, total iron concentrations consistently matched the initial flush of ferrous iron into solution. This precluded the possibility that the rate of leaching out of ferrous iron was greater than the oxidizing ability present and hence was a net effect. At pH 1.4 the small production of ferric iron that would have come into solution in this case was not observed, and not because of precipitation, given the high initial acidity. With control leaches using the same mineral substrates, metal dissolution could not be as extensively achieved in acid medium alone in the absence of bacteria. Neither was the carry over of ferric iron in the inocula (equivalent to less than 1g per litre) sufficient to chemically leach out the ferrous iron in the concentrations observed. By implication, the observed dissolution was bacterial, supported by continued drops in pH value,despite the observation of an apparent absence of iron oxidation. The mechanism for this mineral dissolution could have been preferential sulphide oxidation either because of, or during, inhibition of iron oxidation. (This would be true even if some ferric iron was formed but not observed due to reduction by the mineral sulphide). After the spike of ferrous iron was oxidised, further additional concentrations of ferrous iron were rapidly oxidised, despite the substantial concentrations of sulphide still remaining, in some experiments (fig 6.5). The lack of a lag phase in the oxidation of this supplementary iron did not therefore indicate either prevention of iron oxidation or preferential oxidation of the sulphide, at this point of the batch leach. A possible factor in this observation could have been cell growth. As sulphide oxidation progressed releasing both copper and sulphate,the cell numbers would have increased (this was not measured). If the limitation of iron oxidation was a function of cell number then additions of biomass should reduce the

concentrations of ferrous iron produced. Biomass additions of Sulfolobus BC65 did not affect batch leaching curves (and in one case reduced % extraction). However, the failure of growth with washed cell suspensions during physiological studies implied the death of cells due to centrifugal harvesting (3.3). Oxidation in the oxygen electrode also did not predict the continued ability of (dead) cell suspensions to do so in stirred mineral leach environments.

The release of ferrous iron from copper sulphides contrasted with that observed with pyrite. Ferrous iron from pyrite was only oxidized near the completion of the leach whereas copper extraction could extend beyond the point of iron oxidation. (Extended copper release could have come from the sudden appearance of ferric iron in solution after this ferrous iron was oxidized). The maintenance of an almost constant concentration of ferrous iron, once in solution during pyrite leaching suggested an equilibrium in solution. As the total iron level increased, indicating continued ferric iron production it was possible that the ferrous iron was continually "turned over". If it was an equilibrium function the ferrous iron concentration might have been expected to rise with the total iron concentration. However, total iron leached did not necessarily correspond with levels in solution. Whether precipitated iron could influence an effective ferrous/ferric iron ratio was not investigated. The addition of large concentrations of ferrous iron during pyrite leaching, altering the iron species ratio, was followed by it's rapid removal (fig 6.6). This removal consistantly occurred at two distinct rates. Levelling off of iron oxidation into a slower rate, but at a ferrous iron concentration above that recorded prior to the addition indicated a higher absolute value for the proposed equilibrium level because of the massive increase of total ferric iron in solution. An intermediate level for the cut-off point in the two rates, on adding a smaller concentration of ferrous iron, though supportive of this idea was indeterminate given the fluctuating level of ferrous iron concentration, particularly close to the

limitation of the sulphide leach.

Ferrous iron in solution at a constant level during pyrite dissolution despite continued ferric iron production was analagous to residual ferrous iron in solution during continuous iron oxidation at increasing values of D for T.ferrooxidans (4.4). That is, it may have been a function of the inherent iron oxidation kinetics of the thermophile, indicating a high effective K_s in batch mineral culture for ferrous iron. Apparent K_m values for cell suspensions were closely related to those in chemostat culture for mesophiles. Continuous mineral oxidation at 68°C may have indicated Sulfolobus kinetics similar to those in the oxygen electrode. The assumption was that ferrous iron levels could be significantly lowered during continuous oxidation. This assumed control of oxidation by the ferric/ferrous ratio, but did not explain the leaching of copper ores unless the ferrous iron itself was limiting (until the cell biomass increased, argued above). Support of this possibility would be provided if the lag in ferrous iron oxidation could be extended by further additions of ferrous iron during the initial stages as opposed to after oxidation of the first flush of ferrous iron. Additional to this would be the influence of concentrated biomass, if harvested by a method proven to retain cell viability.

In conclusion therefore, metal release was influenced by iron oxidation at high temperature, the control of which appeared to be by the iron itself, although this was not proved nor measured. Evidence towards this would depend on the answers to the points outlined above.

7 SUMMARY AND CONCLUSIONS

A range of acidophilic iron oxidizing bacteria were compared relative to their iron oxidizing abilities. Isolates capable of iron oxidation at moderately thermophilic temperatures (50°C) were shown not to have a universal obligate requirement for reduced sulphur as an assimilatable sulphur source (3.2). This contrasted with previous studies using the same isolates (1.8.2). Therefore, strain TH3 was confirmed as an obligate chemolithotrophic heterotroph, incapable of mixotrophic growth with glucose. The essential growth requirement provided by yeast extract remained undefined. Strains BC1, TH1, ALV and LM2 were confirmed as facultatively autotrophic but the addition of reduced sulphur was shown still to be a requirement during mixotrophic growth, except for strain ALV. Growth of strain ALV was the first indication amongst these isolates of the ability to use sulphate as the sole sulphur source. Sulfolobus BC65 also required reduced sulphur during iron oxidation. The sulphur requirement for all the strains could be met by thiosulphate, tetrathionate or elemental sulphur. Both strain ALV and T.ferrooxidans, which did not require reduced sulphur were partially inhibited in the presence of thiosulphate. Growth curves indicated that the quantitative requirement was at least an order of magnitude less with strain LM2 than strains TH1 and BC1. Additionally, the comparison of strains TH1/BC1 provided physiological evidence that these were similar isolates (confirmed by gel electrophoresis and DNA homology studies, Norris et al, 1988).

Cell suspensions of all the studied bacteria exhibited ferrous iron oxidizing activity in the oxygen electrode. Oxidation was, without exception, inhibited competitively by ferric iron. Each strain was measured for a specific value of apparent K_m for ferrous iron calculated from several graphical methods and K_i for ferric iron inhibition (tables 4.7; 4.8). Values were organism

specific as were the individual traces measured during oxidation. Maximum observed rates were delayed by accelaration phases (previously observed with T.ferrooxidans. 1.7.1) the length depending on organism and substrate/inhibitor concentrations. The apparent K_m values of 1.34 mM and 0.25 mM for T.ferrooxidans and L.ferrooxidans respectively were at the higher and lower ranges previously reported for these organisms (1.7.1; 1.8.1). The value for strain TH1, however, of 1.03 mM ferrous iron contrasted sharply with the only other previously reported value for TH1 of 7.3 mM (1.8.2). Strains TH3, LM2, ALV and Sulfolobus BC65 were in order, 0.45 mM, 1.525 mM, 2.96 mM and 0.56 mM (4.2) for apparent K_m. With Cu as an inhibitor the greater sensitivity of L.ferrooxidans than T.ferrooxidans was confirmed but the form of inhibition with Leptospirillum was shown to differ, being uncompatitive in form (4.2.6).

Growth curves on ferrous iron, differing in the growth rate of each organism also differed in response to ferric iron. These observations could be related to K_i values for the cell suspensions. L.ferrooxidans. with the highest K_i of 37.9 mM ferric iron showed identical oxidation of 25 mM ferrous iron in the presence or absence of 125 mM ferric iron (4.3.1). In comparison, strain ALV with the lowest K_i of 1.02 mM ferric iron did not grow in the presence of the ferric iron (4.3.2). Within the experimentally measured values of apparent K_m and K_i, there appeared to be evidence for a ratio between both constants that was organism specific. No physiological trait was though directly attributable to this observation.

Growth and iron oxidation during continuous culture displayed features that could be related to cell suspension kinetics. The shape of batch growth curves and response to ferric iron of strain ALV predicted it's poor response to ferric iron in solution. During continuous culture this organism progressively decreased in the percentage iron oxidized as dilution rate was increased for a constant limiting ferrous iron substrate concentration (4.4.3). Stressed at all flow rates this

organism achieved maximum oxidation of 44 mg per h ferric iron compared to 127 mg per h ferric iron by T.ferrooxidans in comparable culture conditions. The limit for sustainable growth of strain ALV appeared to be at a D approaching 0.08 h^{-1}.

The value of D controlled the relative ratios of T.ferrooxidans and L.ferrooxidans in mixed continuous culture (4.4.4). Leptospirillum cells predominated at low flow rate but decreased in relative numbers as flow rate increased. This was predicted by the higher affinity for ferrous iron as measured by the lower apparent K_m for L.ferrooxidans than T.ferrooxidans. The percentage iron oxidized was always greater by a few percent at low D in monocultures of L.ferrooxidans than of T.ferrooxidans.

The influence of inherent affinities during competation was offset by the aggregation of Leptospirillum cells. These lessened the impact of flow rate on washout. By deliberately encouraging cell aggregation in pure culture, productivity values of ferric iron were increased (4.4.1). This was the first example of enhanced iron oxidation by increasing biomass both without an inert support, cell carrier or recycle and with an organism other than T.ferrooxidans. The maximum production achieved was 490 mg per h ferric iron. This compared well with a Bacfox type test with T.ferrooxidans where although maximum production was 602 mg per h ferric iron the sustainable production was estimated at 500-550 mg per h ferric iron (4.4.2). This was only sustainable at pH values that allowed iron precipitation. Leptospirillum aggregated in the absence of any precipitates.

The relevance of iron oxidation and it's kinetics in mineral oxidation was concentrated in studies on Sulfolobus BC65. Investigation of leaching in shake flasks became impractical in differentiating between small metal concentrations released by changes in experimental conditions. Conditions that increased metal dissolution at 68°C were reduced particle size and adequate provision of carbon dioxide (figs 6.2; 6.3; 6.4). Release of copper in solution was preceded by ferrous iron. Quicker

oxidation of this substrate may have increased potential yields. The implication from experimental data was that levels of iron (of both species) controlled iron oxidation (6.2). That is, ferrous iron levels were initially inhibitory during copper dissolution and that the ferric to ferrous ratio was controlling in pyrite dissolution.

A qualitative comparison of the acidophiles indicated differences between them in respect of the components of their individual respiratory chains. Based on the wavelengths of peaks in whole cell spectra at room and liquid nitrogen temperature, T.ferrooxidans, L.ferrooxidans, Sulfolobus BC65 and strains TH1 and ALV all had b and c type cytochromes (5.2). Each organism also indicated the presence of an oxidase but Leptospirillum alone showed no evidence of an a/aa_3 type cytochrome. Strains ALV and TH1 were very similar to each other but different from T.ferrooxidans. On this evidence each organism (with the possible exception of the moderate thermophiles) appeared to utilize separate biochemistry in the utilization of inorganic energy sources. The greatest differences were with L.ferrooxidans and Sulfolobus BC65 with absorbance maxima at 578 nm and 576 nm respectively. Not attributable to known cytochrome species the absorbance of the Leptospirillum was attributable to a red coloured, acid stable protein. This was reduced by ferrous iron and did not form a pyridine hemochromogen of known heme groups.

This study showed that the physiology of the moderate thermophiles had not been fully elucidated. The relevance to the ecology of the organisms was not shown. However, in this context it would be significant if strain TH3 could obtain it's growth requirement from the cell leakage or growth products of other isolates from heterogeneous environments. Heterogeneity was also seen in the widespread values of apparent K_m. Environmentally, competition between organisms would also be influenced by these relative values as well as response to pH, temperature, nutrients and inhibitors.This influence of kinetics may explain observed domination of Leptospirillum cells over Thiobacilli in pyrite enrichment cultures

(Norris, 1983) although as well as indicating differences in the acidophiles the continuous oxidation of iron showed the potential of Leptospirillum cells in iron regeneration via the altered kinetics of cell aggregates. This removed the isolation of T.ferrooxidans as the only iron oxidizer worthy of consideration in such operations.

The inhibitory effect of ferric iron to growth appeared to be partly predictable by the value of measured K_i for an organism. The K_i for strain LM2 if measured would support this as it's response to ferric iron in batch growth predicted that the value should be less than that for strain ALV. Similarly continuous culture of Sulfolobus BC65 on mineral would be expected to show reduced concentrations of ferrous iron in solution. The manipulation of iron release from batch culture by adding biomass and ferrous iron would also show whether control of iron oxidation was at the level of the concentration of the iron itself.

Differences in the acidophiles extended to the components of their respiratory chains. Study of the unknown absorbances of Sulfolobus and Leptospirillum would indicate whether these separate species contain similar enzymes or at least proteins with a comparable prosthetic group and it's identity.

Therefore this study reports information in partial satisfaction of the aims of the project (1.9); with indications towards further points of investigation in order to extend understanding of acidophilic iron oxidation.

APPENDIX 1

SOURCES AND PREPARATION OF MINERAL SUBSTRATE

Several sources of mineral sulphide were used, both as energy substrates in liquid culture and for use in comparison of growth with different mineral types using a range of organisms. The particular mineral sample used at any one time for growth and stock cultures reflected abundance of short term supply of a particular mineral sample, that could be shown by test growth curves to be capable of supporting acidophilic growth. Mineral sulphides were supplied as ore in various stages of preparation, from crushed rock to ex-flotation concentrates. Large sized samples or ground samples that had agglomerated were reduced in size by wet grinding in a rotary ball mill. This ground material was then passed through a series of metal sieves of pre-determined pore size. The mechanical operation of these sieves ultimately limited the separation of the finest particle sizes. Each sized portion was kept dry in sealed glass or plastic containers and representative samples were routinely ground by spatula to remove obvious (small) lumps before addition to liquid media. On addition to media, oil-like films appeared on the surface, particularly with ex-flotation concentrates. Such films were probably caused by residual flotation agents used in previous mineral processing. Their effect on growth was not investigated and may have been the cause of failed growth of some strains on particular samples, expected to support growth. However, such failure of growth was more likely caused by different structures of samples which were grouped under one ore type eg pyrite, especially as pre-washing such ore did not alleviate this apparent toxicity.

Mechanical sieves were insufficient to provide graded particle sizes below 100 microns. In investigating the potential of mineral dissolution by *Sulfolobus* BC65 as mean particle size became very small (6.1) a series of decreasing particle sizes was prepared of a Spanish copper

concentrate (supplied by and processed at Warren Spring Lab, Stevenage). A slurry was prepared by washing 1 kg of concentrate through a 200 micron sieve. This was pressure filtered and air dried on the filter paper. Approximately ten 50 g portions were re-powdered and individually passed through a cyclosizer. This consisted of passing water under pressure through a descending series of glass cones, shaped to maintain internal vortices. Such density separation maintained a decreasing particle size within each cone. After each run the individual cones were drained and the mineral slurries separately bulked before pressure filtering and hot air drying of the individual size-graded samples. Each preparation was calculated as being within set size bands. This was checked after drying by measuring particle size distribution using a Malvern particle sizer. Measurement was made by passing laser light through a small stirred sample chamber filled with a water suspension of both a few grains of sample and a surface active agent. Each measured sample produced a distribution of particles within size bands. Fig A1.1 represents a copy of the printout for the particle sizes used in the experiment in 6.1 . Sample 1 was the original concentrate filtered through a 100 micron mesh sieve. Samples 2 to 5 were passed through the cyclosizer and all show a much reduced range of particle sizes, relative to sample 1 and all show the mean value of particle size to be decreasing. Despite the decrease in mean particle size the overall size distribution within each finally prepared sample was much larger than that expected .

The several types of mineral used throughout the study represented a world wide distribution of original sources. Pyrite ores from Spain, Swedan and South Africa were kindly supplied by N W LeRoux, Warren Spring Lab, Stevenage, Dr B Lindstrom, Umea University, Swedan, and Davy McKee Ltd, Stockton. Chalcopyrite based copper concentrates were supplied from the Avoca mine in Eire and from Santiago in Spain (both supplied by N W LeRoux) and from Australia. Nickel ore was supplied by International Nickel Ltd.

MALVERN 2600/3600 PARTICLE SIZER Ver.2.4

MALVERN INSTRUMENTS LTD.SPRING LANE,MALVERN,ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 1 Sample 1

TIME 01-37-38 RUN NO. 17 LOG ERROR = 4.63

SAMPLE CONCENTRATION = 0.0068 % BY VOLUME
OBSCURATION = 0.17

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	HEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
188.0	87.2	99.5	0.5	0.0	283	191
87.2	53.5	98.7	2.8	0.5	342	327
53.5	37.6	96.1	6.6	3.3	519	507
37.6	28.1	79.8	16.4	9.9	715	714
28.1	21.5	66.5	13.2	26.2	989	981
21.5	16.7	56.5	10.0	39.5	1301	1237
16.7	13.0	35.9	14.4	49.7	1646	1647
13.0	10.1	24.1	11.8	64.1	1909	1878
10.1	7.9	12.5	10.5	76.0	2039	2047
7.9	6.2	5.8	6.7	86.5	2047	2041
6.2	4.8	3.0	3.8	93.3	1926	1898
4.8	3.8	0.8	2.2	97.0	1709	1737
3.8	3.0	0.1	0.7	99.2	1474	1509
3.0	2.4	0.0	0.1	99.9	1344	1339
2.4	1.9	0.0	0.0	100.0	1282	1273

HEIGHT OF BIGGEST BAND
BEST LOG ERROR 4.63

MALVERN 2600/3600 PARTICLE SIZER Ver.2.4

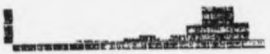
MALVERN INSTRUMENTS LTD.SPRING LANE,MALVERN,ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 2 Sample 2

TIME 01-42-20 RUN NO. 18 LOG ERROR = 4.14

SAMPLE CONCENTRATION = 0.0059 % BY VOLUME
OBSCURATION = 0.09

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	HEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
188.0	87.2	99.4	0.6	0.0	912	905
87.2	53.5	89.4	10.0	0.6	1486	1398
53.5	37.6	52.6	36.8	10.6	1801	1809
37.6	28.1	25.4	27.2	47.4	2021	2019
28.1	21.5	11.6	13.8	74.6	2047	2047
21.5	16.7	11.0	4.6	86.5	1911	1949
16.7	13.0	9.5	2.1	88.4	1594	1597
13.0	10.1	7.0	2.5	90.5	1346	1328
10.1	7.9	2.6	2.5	94.0	1614	1642
7.9	6.2	2.9	0.6	96.5	1697	1671
6.2	4.8	1.8	1.1	97.1	1645	1629
4.8	3.8	0.2	1.7	98.1	1554	1574
3.8	3.0	0.0	0.2	99.8	1531	1517
3.0	2.4	0.0	0.0	100.0	1504	1491
2.4	1.9	0.0	0.0	100.0	1454	1471



WEIGHT ON DIODE SIZES
BEST LOG ERROR = 4.14

MALVERN 2600/3600 PARTICLE SIZER Vn:3

MALVERN INSTRUMENTS LTD,SPRING LANE,MALVERN,ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 3 Sample 3

TIME 01-46-30 RUN NO. 21 LOG ERROR = 5.76

SAMPLE CONCENTRATION = 0.0121 :: BY VOLUME
OBSCURATION = 0.17

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	WEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
100.0	87.2	99.7	0.3	0.0	586	587
87.2	76.0	94.6	5.2	0.3	507	502
76.0	66.3	89.4	5.1	5.5	1373	1391
66.3	57.8	83.3	6.1	10.6	1727	1726
57.8	50.4	76.7	6.6	16.7	2017	2029
50.4	43.9	68.3	8.4	23.3	2047	2047
43.9	38.3	59.1	9.2	31.7	1754	1758
38.3	33.4	49.4	9.7	40.9	1190	1190
33.4	29.1	39.7	9.7	50.6	759	805
29.1	25.4	30.0	9.7	60.3	639	644
25.4	22.1	20.3	9.7	70.0	613	553
22.1	19.3	10.6	9.7	79.7	588	446
19.3	16.8	1.0	9.6	89.4	606	362
16.8	14.7	0.0	1.0	99.0	681	296
14.7	12.8	0.0	0.0	100.0	817	227
12.8	11.2	0.0	0.0	100.0		
11.2	9.7	0.0	0.0	100.0		
9.7	8.5	0.0	0.0	100.0		
8.5	7.4	0.0	0.0	100.0		
7.4	6.5	0.0	0.0	100.0		
6.5	5.6	0.0	0.0	100.0		
5.6	4.9	0.0	0.0	100.0		
4.9	4.3	0.0	0.0	100.0		
4.3	3.8	0.0	0.0	100.0		
3.8	3.3	0.0	0.0	100.0		
3.3	2.9	0.0	0.0	100.0		
2.9	2.4	0.0	0.0	100.0		
2.4	1.9	0.0	0.0	100.0		

PAGE 1.

WEIGHT ON DIODE SIZES
BEST LOG ERROR = 5.76

MALVERN 2600/3600 PARTICLE SIZER Vn:3

MALVERN INSTRUMENTS LTD,SPRING LANE,MALVERN,ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 4 Sample 4

TIME 01-58-08 RUN NO. 23 LOG ERROR = 5.61

SAMPLE CONCENTRATION = 0.0087 % BY VOLUME
OBSCURATION = 0.18

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	WEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
100.0	87.2	100.0	0.1	0.0	266	254
87.2	76.0	99.1	0.9	0.1	452	456
76.0	66.3	95.1	4.0	0.9	786	783
66.3	57.8	89.1	6.0	4.9	1010	1003
57.8	50.4	82.6	6.5	10.9	1398	1390
50.4	43.9	73.9	8.7	17.4	1768	1777
43.9	38.3	63.3	10.6	26.1	2047	2047
38.3	33.4	50.1	13.2	36.7	1956	1960
33.4	29.1	36.9	13.2	49.9	1531	1552
29.1	25.4	23.7	13.2	63.1	1057	1045
25.4	22.1	10.5	13.2	76.3	770	720
22.1	19.3	0.0	10.5	89.5	661	589
19.3	16.8	0.0	0.0	100.0	665	517
16.8	14.7	0.0	0.0	100.0	742	424
14.7	12.8	0.0	0.0	100.0	867	354
12.8	11.2	0.0	0.0	100.0		
11.2	9.7	0.0	0.0	100.0		
9.7	8.5	0.0	0.0	100.0		
8.5	7.4	0.0	0.0	100.0		
7.4	6.5	0.0	0.0	100.0		
6.5	5.6	0.0	0.0	100.0		
5.6	4.9	0.0	0.0	100.0		
4.9	4.3	0.0	0.0	100.0		
4.3	3.8	0.0	0.0	100.0		
3.8	3.3	0.0	0.0	100.0		
3.3	2.9	0.0	0.0	100.0		
2.9	2.4	0.0	0.0	100.0		
2.4	1.9	0.0	0.0	100.0		

PAGE 1.

UPPER	LOWER	WT BELOW	IN BAND	WT ABOVE	COMPUTED	MEASURED
188.0	97.2	99.7	0.3	0.0	600	597
97.2	93.5	99.4	0.3	0.3	907	902
93.5	37.6	76.7	17.8	5.4	1379	1301
37.6	28.1	46.3	30.4	23.3	1727	1726
28.1	21.5	15.8	30.5	53.7	2017	2029
21.5	16.7	2.6	13.2	84.2	2047	2047
16.7	13.0	0.4	2.2	97.4	1754	1758
13.0	10.1	0.0	0.4	99.6	1190	1190
10.1	7.9	0.0	0.0	100.0	759	805
7.9	6.2	0.0	0.0	100.0	635	644
6.2	4.8	0.0	0.0	100.0	513	553
4.8	3.8	0.0	0.0	100.0	388	446
3.8	3.0	0.0	0.0	100.0	606	362
3.0	2.4	0.0	0.0	100.0	661	296
2.4	1.9	0.0	0.0	100.0	317	227

WEIGHT OU DIODE SIZES
BEST LOG ERROR = 5.76

MALVERN 2600/3600 PARTICLE SIZER V4.2

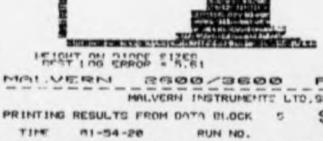
MALVERN INSTRUMENTS LTD,SPRING LANE,MALVERN,ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 4 Sample 4

TIME 01-58-08 RUN NO. 23 LOG ERROR = 5.61

SAMPLE CONCENTRATION = 0.0087 % BY VOLUME
OBSCURATION = 0.19

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	WEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
188.0	97.2	100.0	0.1	0.0	266	259
97.2	93.5	99.9	0.1	0.1	412	400
93.5	37.6	95.1	4.8	4.9	786	783
37.6	28.1	83.8	11.3	16.2	1010	1003
28.1	21.5	59.9	23.7	40.1	1398	1396
21.5	16.7	28.8	30.6	71.2	1768	1778
16.7	13.0	10.1	18.2	89.9	2047	2047
13.0	10.1	1.9	8.2	98.1	1956	1920
10.1	7.9	0.1	1.8	99.1	1531	1552
7.9	6.2	0.0	0.1	99.9	1057	1045
6.2	4.8	0.0	0.0	100.0	776	725
4.8	3.8	0.0	0.0	100.0	661	605
3.8	3.0	0.0	0.0	100.0	605	417
3.0	2.4	0.0	0.0	100.0	742	424
2.4	1.9	0.0	0.0	100.0	352	324



WEIGHT OU DIODE SIZES
BEST LOG ERROR = 5.61

MALVERN 2600/3600 PARTICLE SIZER V4.2

MALVERN INSTRUMENTS LTD,SPRING LANE,MALVERN,ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 5 Sample 5

TIME 01-54-20 RUN NO. 25 LOG ERROR = 5.52

SAMPLE CONCENTRATION = 0.0069 % BY VOLUME
OBSCURATION = 0.17

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	WEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
188.0	97.2	99.9	0.1	0.0	184	184
97.2	93.5	99.8	0.1	0.1	322	318
93.5	37.6	96.6	3.2	3.4	683	678
37.6	28.1	86.4	10.2	13.6	940	918
28.1	21.5	65.6	20.8	34.4	1348	1345
21.5	16.7	29.4	36.2	70.6	1869	1891

2.4	1.9	0.0	0.0	100.0	353	324

PAGE

HEIGHT ON DIODE SIZES
BEST LOG ERROR = 4.61

MALVERN 2600/3600 PARTICLE SIZER V2.3

MALVERN INSTRUMENTS LTD, SPRING LANE, MALVERN, ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 5 Sample 5

TIME 01-54-20 RUN NO. 25 LOG ERROR = 5.52

SAMPLE CONCENTRATION = 0.0059 % BY VOLUME
OBSCURATION = 0.17

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	WEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
188.0	97.2	99.9	0.1	0.0	184	184
97.2	53.5	99.8	0.1	0.1	323	308
53.5	37.6	99.6	0.2	0.3	503	510
37.6	28.1	98.0	1.6	1.9	740	745
28.1	21.5	93.4	4.6	6.5	1069	1081
21.5	16.7	51.0	24.4	24.5	1455	1472
16.7	13.0	26.6	24.4	49.0	1851	1878
13.0	10.1	9.5	19.5	72.0	2047	2047
10.1	7.9	6.4	3.1	96.5	1989	1978
7.9	6.2	6.0	0.4	99.6	1855	1853
6.2	4.8	0.0	0.0	100.0	1163	1032
4.8	3.8	0.0	0.0	100.0	778	787
3.8	3.0	0.0	0.0	100.0	703	704
3.0	2.4	0.0	0.0	100.0	766	561
2.4	1.9	0.0	0.0	100.0	670	403

PAGE

HEIGHT ON DIODE SIZES
BEST LOG ERROR = 5.52

Fig A1.1
Printout of size distribution for a series of preparations of a ground chalcopyrite concentrate separated by density values of the individual particles in water in a Warman cyclosizer. The relative concentrations (%) of ore particle size within defined boundaries (to 0.1 of a micron) are presented in comparative histograms for five sampled preparations. Sample 1 represents size distribution within the original ground copper concentrate after passage through a mechanical sieve of 100 micron size. The subsequent samples display narrower ranges of size distribution due to the separation process and a decrease in the mean particle size for each subsequent preparation. These laser measured readings represented the most accurate method of confirming particle size separation. (The effect of these particle size preparations on metal dissolution is shown in fig 6.3)

APPENDIX 2

CALCULATION AND USE OF OXYGEN CONCENTRATION IN THE OXYGEN ELECTRODE

Calibration of the oxygen electrode could not be directly specified in units of oxygen consumed over time due to the acidic nature of the medium and high incubation temperatures used (2.4.1) Oxygen content of the medium was therefore based on figures of solubility for oxygen in water. The figures used were derived from data, summarized in table A2.1, adapted from "Solubility of inorganic and organic compounds", tables 22, 23, and 25 (Stephen and Stephen, Pergamon Press, 1963).

TABLE A2.1

Solubility constants for molecular oxygen in water, showing their conversion to specific concentration values, allowing both for the influence of temperature and the reduced oxygen content in an air saturated solution.

Bunsen solubilities (O_2 in H_2O)

Liquid temp (°C)	ex Table 22	ex Table 23	Average x1000 (ml $O_2 l^{-1}$)	O_2 content at atm-conc*	specific conc#
5	0.0439	0.0429	43.40	9.092	406
10	0.0390	0.0380	38.50	8.066	360
20	0.0317	0.0310	31.35	6.568	293
30	0.0268	0.0261	26.45	5.541	247
40	0.0233	0.0231	23.20	4.860	217
50	0.0207	0.0209	20.80	4.358	195
60	0.0189	0.0195	19.20	4.022	180
70	0.0178	0.0183	18.05	3.781	169
80	0.0172	0.0176	17.40	3.645	162
90	0.0169	0.0172	17.05	3.572	159

* Conversion of solubility for an air saturated solution assuming oxygen concentration at the normal atmospheric value is 20.95% v/v O_2 in air therefore conversion factor x 0.2095 gives ml O_2 per l of water during air saturation.

$^{\beta}$ Assuming 1 μ mol O_2 = 22.4 μl, therefore
y $\frac{\text{ml } O_2 \text{ per l}}{22.4} \times 10^3$ is oxygen concentration as micromoles per litre.

From Stephan and Stephan (1963), for molecular oxygen at pressure of 760 mm Hg, oxygen content of water at 30°C was between 26.1 and 26.8 ml per litre, an average of 26.45 ml per litre (table A2.1). Calculating concentration for 20.95% molecular oxygen gave 5.54 ml per litre, equivalent to 247 μ moles per litre. For air at 760 mm Hg, at 30°C oxygen dissolution was calculated as 234 μ moles per litre. Therefore taking the mean figure the assumption that acidity had a negligible effect gave a dissolved oxygen value of 240 μ moles per litre in air saturated water at pH 1.7. This figure, calculated from the data by Tuovinen and Kelly (1974) was used for all determinations at 30°C. For dissolution values at 45°C and 65°C the curve of Bunsen solubilities against temperature was constructed (figure A2.1) and the relevant solubilities estimated. Repeating the calculation with the relevant data for 45°C and 65°C gave concentrations of 191 μ moles per litre and 162 μ moles per litre respectively. With the electrode empirically calibrated (2.4.1) chart speed could be converted from divisions per min to oxygen content per ml of reaction per min and hence allowing for protein concentration per ml to μ moles molecular oxygen per minute per mg bacterial protein. This only affected values of V_{max}. Unconverted rate values gave the same values for apparent K_m on calculation.
(Thanks are due to Prof D P Kelly for the outline of this calculation).

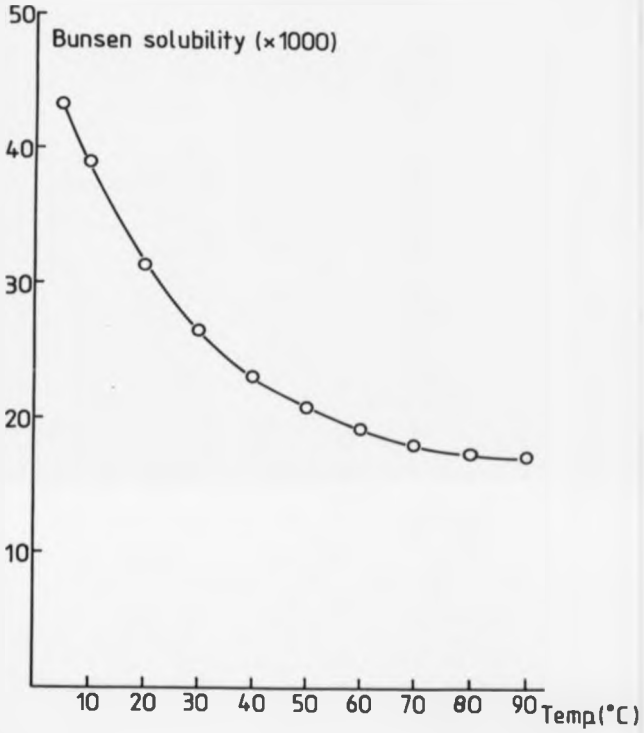


Fig A2.1
Relationship of Bunsen solubility values for molecular oxygen dissolved in water plotted against the water temperature. Plots are drawn from the averaged solubility values, corrected to ml molecular oxygen present per litre of water (table A2.1).

APPENDIX 3

ALTERNATIVE GROWTH DATA FOR THE MODERATE THERMOPHILES. GROWTH CURVES OF STRAIN BC1/TH1 AND COMPARISON OF TEMPERATURE OPTIMUM OF STRAIN ALV AND PUBLISHED DATA

During physiological comparison of the moderate thermophiles both strains TH1 and BC1 were found to be very similar in their growth responses (3.2.2). Taken as evidence of species similarity the data for one or other of the strains was presented in comparison with the other organisms in this study. The corresponding data for the alternative strain only indicated slight differences in rate and extent of growth (figs A3.1 and A3.2). As with strain BC1, strain TH1 was capable of partial iron oxidation in the first sub-culture without reduced sulphur. This oxidation was completed on addition of 1mM glucose (fig A3.1). The iron oxidation rate was similar with either 1mM glucose or 1mM tetrathionate present but only terathionate could sustain this oxidation through serial sub-culture. The concentration of reduced sulphur present was growth limiting, the oxidation of iron increasing in both rate and extent with increased concentration for strain BC1 (fig A3.2). The yields, as iron oxidized, with strain BC1 were similar to strain TH1 (figs A3.2, 3.4). Differences in rate and extent of growth (fig A3.1 cf fig 3.3) were caused in part by a difference of 5°C in the temperature optimum of growth on ferrous iron for these two strains. Strain ALV was found to have the lower optimum value of strain TH1 (fig 3.15). These values could all be compared with known data for a range of iron oxidising acidophiles (fig A3.3). This further highlighted the differences in the physiological responses of the organisms normally grouped together as moderate thermophiles requiring reduced sulphur yet having different temperature optima, responses to reduced sulphur and capacity for iron oxidation (3.5;4.5).

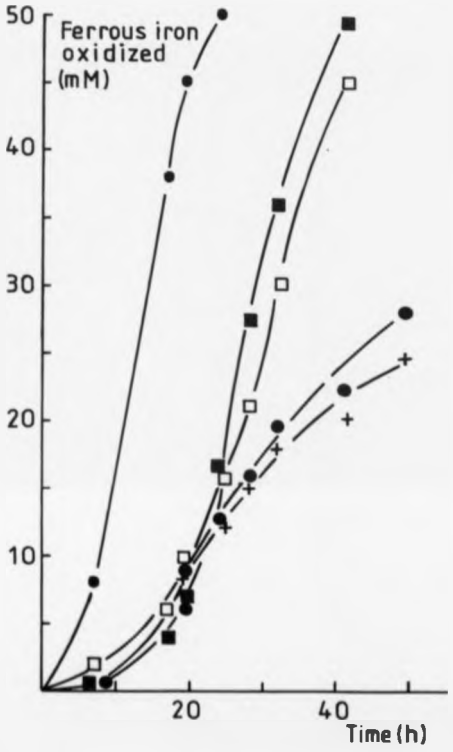


Fig A3.1
Batch growth curves for strain TH1, in 100ml ES at 30°C, pH1.7 in shake flasks(250ml vol) shaken at 120rpm with 50mM ferrous iron, only(+) and plus: $1mgl^{-1}$ YE(●), $100mgl^{-1}$ YE (●)°, 1mM glucose(□) and 1mM tetrathionate(■). Inocula, 5%v/v were from 1mM tetrathionate supplemented culture. Flasks trickled with 1%v/v CO_2 in air except * - diffusion only. (Data for strain BC1 given in fig 3.3).

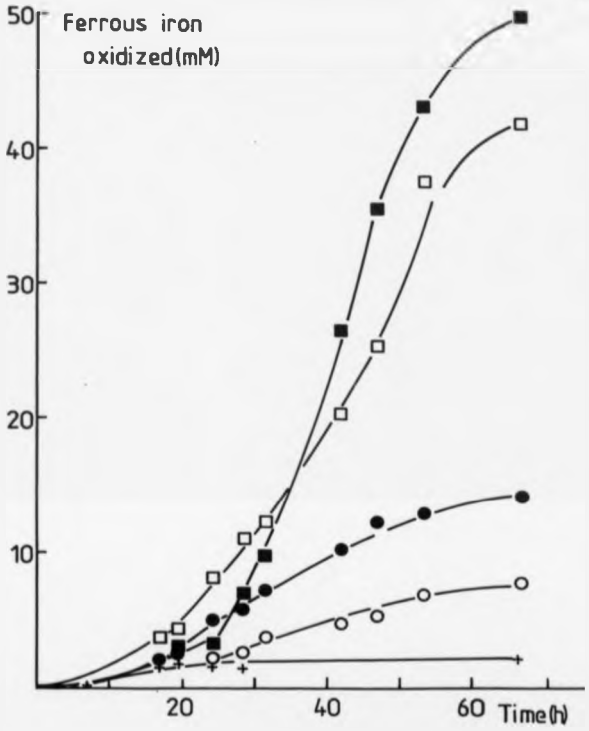


Fig A3.2
Effect of increasing tetrathionate concentration on batch growth curves, as concentration of ferrous iron oxidized over time, of strain BC1. Shake flasks(250ml vol) shaken at 120rpm and 30°C contained 100ml ES at pH1.7. Substrate was 50mM ferrous iron and medium was aerated with 1%v/v CO_2 in air. Flasks contained 0μM(+, identical to sterile control, not shown) 1μM(○), 10μM(●). 100μM(□) and 500μM(■) tetrathionate. Inocula, 3.5%v/v were washed by centrifugation (cf data for strain TH1, fig 3.4).

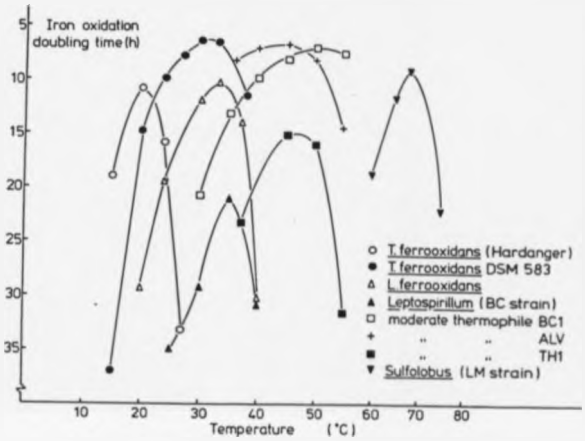


Fig A3.3
Profile of strain ALV (cf fig 3.15) as doubling time of ferrous iron oxidised against growth temperature during batch culture compared with a range of acidophilic iron oxidisers. These include the two mesophilic type strains and two other moderately thermophilic strains used in this study. The strain of Sulfolobus shown differs from that used but indicates the separation of this group from any of the other organisms on the basis of the temperature of optimum iron oxidation. The temperature optima for strains ALV and TH1 were 5°C less than for strain BC1. This differentiated between strains TH1 and BC1 despite their physiological similarities (compare the data of this appendix with chapter 3). The values for T.ferrooxidans DSM 583 and L.ferrooxidans were used to determine the compromise temperature of 32°C during growth of a mixed culture of both organisms (fig 4.36). This graph is reproduced by the kind permission of Dr P.R.Norris, Warwick University.

APPENDIX 4

CALCULATION OF CELL RATIOS FROM INHIBITION DATA BY DIFFERENTIAL EQUATIONS

Using selected concentrations of nitrate and copper (NO_3 and Cu) the mesophiles T.ferrooxidans and L.ferrooxidans displayed differential inhibition of ferrous iron oxidation. Assuming that in mixed cultures total iron oxidation was provided by each organism in the same proportion as cell numbers to each other, this proportion was calculated from values of percentage (%) oxidation (for the mixed culture of 4.4.4). Differential inhibition provided measurable parameters that affected the degree of oxidation. The number (proportion) of T.ferrooxidans cells (and therefore proportion of total activity) was designated X and that of L.ferrooxidans, Y. The rate of mixed iron oxidation was equal to X + Y. Samples were measured with 50 mM ferrous iron and rates calculated specifically for protein concentration. Activity was recorded as degree of oxidation in the presence of inhibitors relative to oxidation of ferrous iron only (the 100% value). Individual components of mixed cultures were assumed to be proportionately inhibited by the same degree as pure cultures. (Subsequent figures were supported by microscope observations,4.4.4). Equations were constructed for each set of samples based on the data of table 4.6.

TABLE 4.6 (in part; figures are % iron oxidation against unihibited controls, for the sample indicated).

Sample		1	2	3	4
T.ferrooxidans	Cu	78	89	100	100
	NO_3	46	33	30	43.7
L.ferrooxidans	Cu	32	49	45	50.3
	NO_3	100	64	64	91.5
Mixed Culture	Cu	32	77	44	76.8
	NO_3	95	57	76	89.3

With sample 1, activity (R) for the mixed culture with Cu

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0.80
Replacing Y = 0.80 in 1) gives 76.8 = 100X + 40.24 and X = 0.37, so Y/X is 2.16 is 2.16:1
With all these calculations, total % oxidation as calculated by X + Y did not give 100%. The proportions were taken as being representative.

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